PROMOTION OF MAMMALIAN ANGIOGENESIS BY PLANT-DERIVED SECONDARY METABOLITES

Farzaneh Kordbacheh

Australian National University

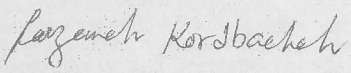
A thesis submitted for the degree of Doctor of Philosophy at Research School of Biology, the Australian National University

May 2013

Declaration

All experimental work presented in this thesis has been performed and analysed by the author unless otherwise stated.

This thesis conforms to The Australian National University guidelines and regulations. The work presented in this thesis has not been submitted for the purpose of obtaining any other degree at this or other universities.



Farzaneh Kordbacheh (Author)

Division of Plant Sciences
Research School of Biology
The Australian national University
May 2013

Acknowledgment

I would like to record my sincere gratitude to the many people who contributed to the production of this thesis. The heartiest and special thanks go to my supervisory panel, Associate Professor Michael Djordjevic, Professor Christopher Parish and Dr Charles Hocart. I would like to thank Michael for giving me the opportunity to join his group at the Research School of Biology (RSB) and to work on a collaborative project with Chris at The John Curtin School of Medical Research (JCSMR). I am also grateful to Michael for the support, interest, guidance and discussions throughout my PhD. I thank Chris for the privilege of working under his supervision. Chris has been an invaluable source of knowledge, intellectual advice, encouragement and support during my PhD. Thanks also to Charles who has been a constant source of support and enthusiasm during my entire PhD in addition to sharing his wealth of knowledge of mass spectrometry and separation chemistry. Lessons I learnt during my PhD project extended beyond matters of science. My supervisors also taught me how to be a more understanding and caring scientist.

I am also grateful for the guidance and technical support provided by Ms Anna Bezos during this project, especially in the time that I spent at JCSMR, with the angiogenesis bioassays and cellular mode of action studies. I would also like to express my appreciation to Mr Thomas Carruthers and Professor Gottfried Otting at the Research School of Chemistry for their contributions to the structural elucidation of ***FK1*** and ***FK2*** by NMR. I would also like to thank Associate Professor Alex Maier and his PhD student, Mr Phuong Tran, for conducting the anti-malaria bioassays with ***FK1***. I am appreciative of the technical assistance and friendly help of Susanti for sharing her experience of human cell culturing, Dr Charles Buer for sharing his flavonoid knowledge and materials, Dr Farid Rahimi for his assistance with the IncuCyte incubator and statistical analysis, Dr Hancai Chen for his help with culturing soybeans and optimising the extraction of xylem sap, Ms Marie Oakes and Ms Lauren Du Fall for their initial work on the analysis of soybean sap metabolites and the discoveries that ultimately led to this project, Ms Anna Brown for sharing her cell lines and finally, Ms Cathy Gillespie for her help with the microscope work.

This project would not have been possible without the financial support of The Australian National University and my supervisors, Michael and Chris, who provided

an ANU PhD scholarship. I also thank Dr Charles Hocart and Dr Hilary Stuart-Williams for the opportunity to work as their technical assistants in both the Mass Spectrometry Facility and Stable Isotope Laboratory, respectively, as well as sharing a series of pleasant conversations over lunches and tea breaks with them and Dr Thy Truong.

I extend my gratitude to all my colleagues and friends in both the Division of Plant Sciences at RSB and the Cancer and Vascular Biology Group at JCSMR.

And last, but definitely not least, I am greatly indebted for the love and support I have received from my family, particularly my Mother and Father, who over all these years and despite being on the other side of the world, have always been there when I needed them.

List of Abbreviations

[^{3}H]-thymidine	tritium labelled-thymidine
1/2D	1/2 dimensional
Ac	acetyl
Akt	v-akt murine thymoma viral oncogene
AMD	age-related macular degeneration
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
ANU	Australian National University
AR	androgen receptor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
C4H	cinnamate 4-hydroxylase
CAD	cinnamyl alcohol dehydrogenase
CAM	chorioallantoic membrane
Cb	carbamoyl
CBG	cytosolic β-glycosidase
CID	collision induced dissociation
CILR	Centre for Integrative Legume Research
CLESH-1	CD36 LIMPII Emp structural homology domain-1
CLIBS	cation-and-ligand influenced binding site
COX-2	cyclooxygenase-II
CTGF	connective tissue growth factor
cv	cultivar
D_2O	deuterium oxide
Da	dalton
DAG	diacylglycerol
dd	doublet of doublets
DHF	4′, 7-dihydroxyflavone
DHK	dihydrokaempferol
DHP	dehydropolymerase
DHQ	dihydroquercetin
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide

DQF-COSY	double quantum filtered- correlation spectroscopy
dt	doublet of triplets
DTT	dithiothreitol
EC	endothelial cell
ECGF	endothelial cell growth factor
ECGS	endothelial cell growth supplement
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EHS	Engelbreth-Holm-Swarm
EPO	erythropoietin
ERβ	oestrogen receptor
ESAF	endothelial cell stimulating angiogenesis factor
ESI	electrospray ionisation
F5H	ferulate 5-hydroxylase
FAK	focal adhesion kinase
FDA	USA Food and Drug Administration
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Flt	Fms-like tyrosine kinase-1
FRS2α	fibroblast growth factor receptor substrate 2α
Fuc	fucosyl
GAB1	GRB2-associated binding protein 1
GC/MS	gas chromatography mass spectrometry
G-CSF	granulocyte-colony-stimulating factor
GRB2	growth factor receptor-bound protein 2
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
HGF/SF	hepatocyte growth factor/scatter factor
HIF-1α	hypoxia-induced factor-1α
HIFCS	heat inactivated foetal calf serum

HMBC	heteronuclear multiple bond coherence
HMBC	heteronuclear multiple bond correlation
HMEC	human microvascular endothelial cell
HO-1	haeme oxygenase-1
HPLC	high performance liquid chromatography
HRG	histidine-rich glycoprotein
HRR domain	histidine-rich region
HSPG	heparan sulfate proteoglycans
HSQC	heteronuclear single quantum coherence
HUVEC	human umbilical vein endothelial cells
IC50	half maximal inhibitory concentration
IFN	interferon
IL	interleukin
IP-10	interferon-inducible protein-10
IP3	inositol 1,4,5-triphosphate
JCSMR	John Curtin School of Medical Research
JNK	c-Jun N-terminal kinase
kDa	kilodaltons
LC/MS	liquid chromatography mass spectrometry
LCO	lipo-chitooligosaccharides
LDL	Low-density lipoprotein
Log *P*	partition coefficient
LPH	lactase-phlorizin hydrolase
m/z	mass to charge ratio
mAb	monoclonal antibody
MadCAM-1	mucosal vascular addressin cell adhesion molecule-1
MAPK	mitogen activated protein kinase
MCP-1	macrophage chemoattractant protein-1
Me	methyl
min	minute(s)
MLEV	Malcolm Levitt's CPD sequence
MMP	matrix metalloproteinase
M_r	molecular weight
MS	mass spectrometry
mTOR	mammalian target of rapamycin

nAChR	nicotinic acetylcholine receptors
NFkB	nuclear factor kappa B
NMR	nuclear magnetic resonance
Nod factor	nodulation factor
NOESY	nuclear overhauser enhancement spectroscopy
NOS	nitric oxide synthase
NRP	neuropilins
OD	optical density
OMT	*O*-methyltransferase
OT	oxytocin
P	p value
PA	Plasminogen activator receptor
PAI-1	Plasminogen activator inhibitor-1
PAL	phenylalanine ammonia-lyse
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PDA	photodiode array
PD-ECGF	platelet-derived endothelial-cell growth factor
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PECAM1	platelet/endothelial cell adhesion molecule-1
PEDF	pigment epithelium derived factor
PI3K/Akt	phosphoinositide 3-kinase/anti-apoptotic kinase
PIGF	phosphatidylinositol-glycan biosynthesis class F protein
PIP2	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PLCγ	phospholipase Cγ
PlGF	placental growth factor
ppm	parts per million
PRP	pattern recognition receptors
PSA	polar surface area
Psi	pound per square inch
PSN	penicillin, streptomycin and neomycin
QED	quantitative estimate of drug-likeness
QTOF	quadrupole time-of-flight mass spectrometer

RARM	rat aorta ring model
RAS	rat sarcoma
RCC	renal cell carcinoma
RSB	Research School of Biology
RTK	receptor tyrosine kinase
SAR	structure-activity relationship
SDF-1α	stromal cell-derived factor-1α
SEC	size exclusion chromatography
SEM	standard error of the mean
S-Flt-1	soluble fms-like tyrosine kinase-1
SFM	serum free media
SP 1	specificity protein 1
SOS	son of sevenless
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
Tie-2	tyrosine kinase with immunoglobulin-like and EGF-like domains-2
TIMP	tissue inhibitor of matrix metalloproteinases
TK	tyrosine kinase domain
TLR	toll-like receptors
TNF	tumour necrosis factor
TOCSY	total correlation spectroscopy
Trx-1	thioredoxin-1
TSP-1	thrombospondin-1
TSP-2	thrombospondin-2
U-II	urotensin-II
UNSW	University of New South Wales
u-PA	urokinase plasminogen activator
u-PAR	urokinase plasminogen activator receptor
UV	ultraviolet
VCAM-1	vascular cell adhesion protein-1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VEGI	vascular endothelial growth inhibitor

Abstract

Historically, plant natural products and their derivatives have been invaluable sources of therapeutic agents to enhance human health and to treat disease. Angiogenesis is a complex and highly regulated process of generating new capillary networks from mature blood vessels. Failure to grow blood vessels has been reported to be associated with diseases including stroke, cardiovascular disorders and wound healing. However, pro-angiogenic drugs are less researched than anti-angiogenic drugs. Soybean is a source of nutrition and it contains a number of small metabolites that confer many beneficial health effects. Some of these metabolites have been found to modulate angiogenesis in animal tissue. Therefore, soybean-derived secondary metabolites and their potential therapeutic applications were the focus of this thesis.

In chapter 3, a bioassay-directed discovery approach utilising size exclusion and liquid chromatography enabled the isolation and purification of a number of bioactive fractions from soybean xylem sap. Using high resolution accurate mass spectrometry and NMR, the structure of two pro-angiogenic molecules were elucidated and shown to be *erythro*-guaiacylglycerol-8-*O*-4'-coniferyl alcohol (***FK1***), and *threo*-guaiacylglycerol-8-*O*-4'-coniferyl alcohol (***FK2***). Independently sourced samples of ***FK1*** and ***FK2*** exhibited comparable pro-angiogenic activity to the soybean molecules.

The mode of action of these molecules was investigated in chapter 4 by studying their effect on endothelial cell proliferation, migration, tube formation and adhesion to the extracellular matrix (ECM) components, fibronectin and vitronectin. These compounds enhanced endothelial cell proliferation and endothelial cell tube formation on an artificial ECM, Matrigel, but did not affect endothelial cell migration or adhesion to fibronectin and vitronectin. It is proposed that ***FK1*** and ***FK2*** might enhance angiogenesis via potentiating the potent mitogen, bFGF, and/or its downstream signalling in endothelial cells.

Flavonoids are implicated in positively influencing a number of human conditions and disease by their anti-tumour, anti-inflammatory and anti-oxidant activities. However, the bioactivities of these compounds have been mostly studied at high concentrations (> 1 μM) in *in vitro* and *in vivo* experiments and at these concentrations are physiologically irrelevant. Chapter 5 examined the potential pro-angiogenic activity

of a series of eighteen flavonoids at serum concentrations resulting from a normal diet. Seven flavonoids including genistein and naringenin showed significant promotion of angiogenesis at sub-μM concentrations while genistein inhibited angiogenesis at high μM concentrations. By examining structure-activity relationships we found that pro-angiogenic molecules lack a C2-C3 double bond.

In conclusion, we have explored the potential of plant-derived secondary metabolites to promote angiogenesis in mammals. With novel bioactivity and a possible novel mechanism of action, ***FK1*** and ***FK2*** may have the potential to be developed for the therapeutic treatment of aberrant angiogenesis-related conditions such as cardiovascular disease, ischemia, stroke, chronic wounds and hypertension. Moreover, pro-angiogenic activity of dietary flavonoids, genistein and naringenin, at physiological concentrations has given some insight into the importance of measuring the bioactivity of natural-derived compounds at their physiological concentrations.

Table of Contents

Chapter 1

General Introduction

1.1. Natural products as a source for drug discovery

Natural bioactive products are chemical substances derived from animals, plants or micro-organisms that exert a biological effect on other organisms. These molecules are mostly secondary metabolites and represent large and diverse classes of compounds including macrolides, terpenes, opiates, alkaloids and phenolic acids (Pezzuto 1997; Wase and Wright 2008; Li and Vederas 2009).

Natural products and their derivatives have been an invaluable source of compounds for the pharmaceutical industry. Over 200 years ago (1804), the first pharmaceutically active pure compound, morphine, was isolated from a plant (*Papaver somniferum*) and commercially sold by Merck in 1827 (Hamilton and Baskett 2000). This initiated an era in which drugs came to be identified and isolated from natural sources and later administrated in precise dosage. Later, the discovery of penicillin as an antibacterial filtrate, from the fungi, *Penicillium rubens,* by Fleming in 1928 and its development as a therapeutic by Florey, Chain and Heatley in 1940, revolutionised pharmaceutical research and led to the discovery of other new antibiotics (e.g., tetracyclin and erythromycin) (Li and Vederas 2009), anti-parasitics (e.g., avermecin), anti-malarials (e.g., quinine, artemisinin), lipid control agents (e.g., lovastatin and analogs), immune-suppressants for organ transplants (e.g., cyclosporine, rapamycins) and anti-cancer drugs (e.g., paclitaxel, irrinotecan) (Harvey 2008; Katiyar, Gupta et al. 2012).

A comprehensive review of pharmaceuticals developed over the last 30 years found that 50% of new drugs and 33% of small-molecule drugs were based on natural products or their derivatives (Newman and Cragg 2007; Newman 2008; Newman and Cragg 2012). With respect to anti-cancer and anti-infective agents this proportion is even higher and reaches 60% and 75%, respectively (McChesney, Venkataranan et al. 2007). Indeed, natural products continue to provide a source of diverse and unique bioactive compounds that promote human health and contribute to the treatment of human disease (Kingston 2011) and in many ways the potential for discovering new compounds with pharmaceutical activity is largely unexplored (Njuguna, Masimirembwa et al. 2012).

1.2. Plant natural products: Botanical therapeutics

Plants in particular have a long history as a source of therapeutics. The approximately 250,000 living plant species contain a much greater diversity of bioactive compounds than any chemical library made by humans (Raskin, Ribnicky et al. 2002). Plant chemicals can be found in all parts of a plant, including the most accessible and abundant organs, the roots, shoots, seeds and leaves (Battle, Kyd et al. 2012). In the early 1900s, before the "Synthetic Era", 80% of all medicines were obtained directly from roots, barks and leaves (McChesney, Venkataraman et al. 2007). For example, the bark of the willow tree (*Salix sp.*) contains salicylic acid and was used by the ancient Greeks and native Americans to treat a wide range of ailments (Rishton 2008). Salicylic acid is the precursor of aspirin, a common over-the-counter analgesic that can also be used as an anti-inflammatory treatment and to inhibit platelet aggregation (Schafer 1995). More recently, the Pacific yew tree (*Taxus brevifolia*) (Wani, Taylor et al. 1971) and the French lilac (*Galega officianalis*) have been the source of the active compounds in the drugs paclitaxel (Taxol®) and the biguanide class of antidiabetic, respectively, used in the treatment of breast cancer (Witters 2001). Other plant derived anticancer drugs include vincristine (Oncovin®) and vinblastine from the Madagascar periwinkle (*Catharanthus roseus)*, and camptothecin from the Happy tree (*Camptotheca acuminata)* (Pezzuto 1997). It has been shown that some 25% of all modern active pharmaceutical compounds are from plant or plant derived natural products (Battle, Kyd et al. 2012).

The success of plant compounds for therapeutic applications is remarkable but probably not unexpected given that plants produce a wide range of chemicals to deter herbivores ranging from small insects to large grazing mammals (Battle, Kyd et al. 2012). Moreover, plants and animals were derived from a common ancestor and have many common biosynthetic pathways, regulatory mechanisms, metabolites and signalling molecules (Kushiro, Nambara et al. 2003). Positive selection during evolution has resulted in plants producing a vast array of secondary metabolites to overcome environmental stress and to defend themselves against predation (Benderoth, Textor et al. 2006). These secondary metabolites are usually non-essential for growth and development but important for long term survival (Howitz and Sinclair 2008).

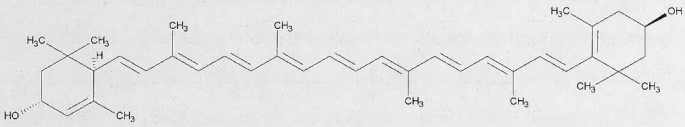
There are many examples of functional conservation between secondary metabolites in plants and animals. The carotenoids, lutein (Fig. 1.1A) and zeaxanthin (Fig. 1.1B) (Mourao, Santana et al. 2009), function in animals to protect the eye from harmful short wave radiation and in plants to protect their photosynthetic machinery from oxidative damage (Demmig-Adams and Adams 2002). These pigments may have evolved from a common biochemical ancestor that played a role in cellular photochemistry (Demmig-Adams and Adams 2002). An additional example of chemical structure conservation is the indole ring found in the regulatory systems of both animals, as melatonin (Fig. 1.1C) (Mourao, Santana et al. 2009) and plants, as auxin (Fig. 1.1D) (Pandi-Perumal, Srinivasan et al. 2006). Auxin is a plant hormone and essential for plant growth. In plants it is involved in processes such as light and gravity responses in root and shoot architecture and vascular development. In animals, auxin has been found to prevent carcinogen induced damage by preventing the down-regulation of anti-oxidant enzymes and the fragmentation of DNA (Mourao, Santana et al. 2009). Melatonin is an animal hormone and is part of apoptotic (programmed cell death) signalling pathways as an anti-oxidant compound. Melatonin has also been found to possess bioactivity in plants by preventing oxidative damage (Pandi-Perumal, Srinivasan et al. 2006).

1.3. General strategies for natural product drug discovery

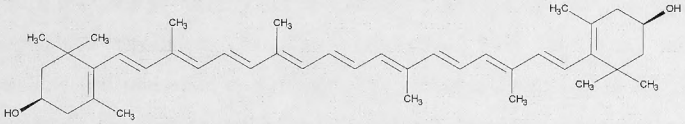
Historically, drugs have been developed from traditional or folk medicines (Holt and Chandra 2002). These may consist of fresh or dry plant material or even simple aqueous or alcoholic extracts. This approach of building on traditional or folk knowledge has enhanced the rate of success in drug discovery in addition to saving time and money (Katiyar, Gupta et al. 2012).

However, in the absence of guidance from traditional practices the key to a successful drug discovery lies in the researchers' ability to identify bioactivity (Piggott and Karuso 2004; Littleton, Rogers et al. 2005; Potterat and Hamburger 2006; Rollinger, Langer et al. 2006). During recent years, many specific and selective bioassays have been developed by which natural products can be evaluated quickly and economically (McChesney, Venkataraman et al. 2007).

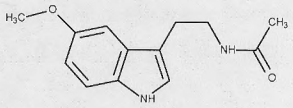
A



B



C



D

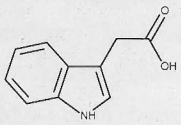


Figure 1.1. Examples of the structurally related secondary metabolites between plants and animals.

Lutein (A), zeaxanthin (B), melatonin (C), auxin (D).

The most efficient method for discovering drugs with novel chemical structure with possibly unique mechanisms of action is bioactivity-guided fractionation (Pezzuto 1997; Kingston 2011). Although in recent years major improvements have been made to improve bioassays it is still necessary to subject extracts to some form of fractionation as crude extracts contain a huge complexity of compounds which may have opposing effects. For example, a crude extract may contain chemicals that inhibit and others that promote particular processes. In addition, there are the added complexities of chemical stability and solubility (McChesney, Venkataraman et al. 2007; Kingston 2011).

Bioassays are used to identify active fractions following one or more orthogonal chromatographic separations targeting properties such as size, solubility, hydrophobicity and ionisation at different pH (Bickerton, Paolini et al. 2012). In a complex mixture, the early passes through the various chromatographic steps will yield active fractions that still represent mixtures of compounds. Ultimately, the purification process must be taken to the point where the active fraction only contains one bioactive compound (Fig. 1.2) (Cordell, Beecher et al. 1991; Pezzuto 1997; Koehn and Carter 2005; Li and Vederas 2009).

The presence of more than one component will create ambiguity as to the identification of the bioactive component. Thus chemical purity must be monitored by additional analyses (e.g., HPLC, GC/MS or LC/MS) until the analyst is certain that structural elucidation can be carried out on the bioactive molecule alone (Butler 2004; Koehn and Carter 2005). The mode of action of the active compound can be studied at this stage but without knowing the structure, it will be a limited investigation. Today, *de novo* structure elucidation is largely based on mass spectrometry (MS) and high field nuclear magnetic resonance (NMR) (Butler 2004; Li and Vederas 2009; Kingston 2011) with the structure finally being confirmed by synthesis and spectroscopic comparison with the isolated material. Then, the mode of action of the active compound can be studied comprehensively (Fig. 1.2).

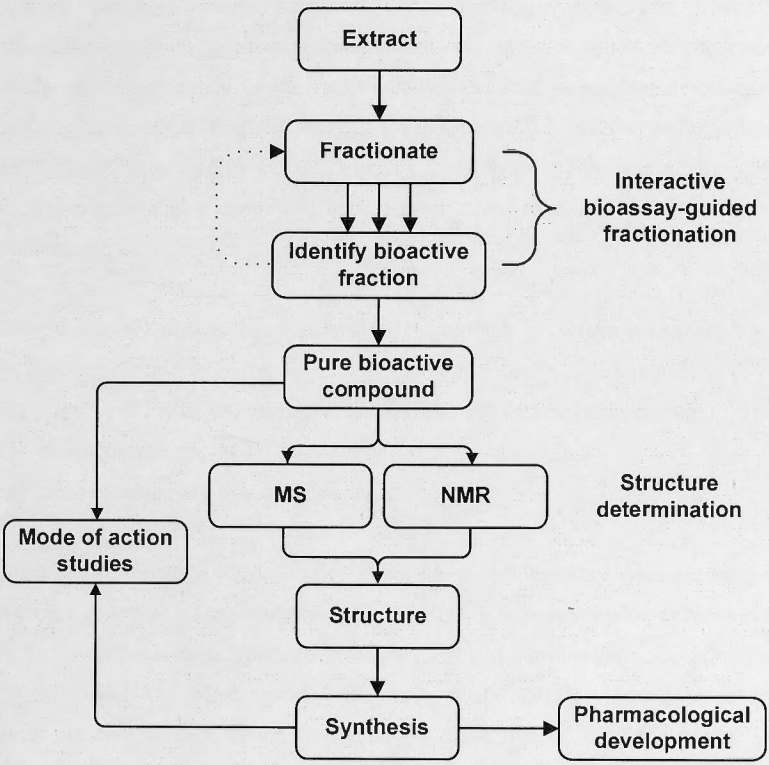


Figure 1.2. General strategy for discovery, *de novo* structure identification of therapeutic compounds and the pharmacological development to a commercial product.

In 1997, Lipinski et al., formulated Lipinski's Rule of Five, also known as Pfizer's Rule of Five or the Rule of Five (RO5), which represented an evaluation of drug-likeness based on properties that would make the compound a likely orally active drug in humans. The rules encompass a set of physico-chemical properties that influence the compound's absorption, distribution, metabolism, and excretion which are important for a drug's pharmacokinetics in the human body. The RO5 represents a strategy for identifying compounds that will have a greater probability of successfully passing clinical trials and getting to market (Lipinski 2004; Leeson and Springthorpe 2007). The rules require that a compound should meet at least three of the four following specifications:

1) number of hydrogen bond donors (HBDs; nitrogen or oxygen atoms with one or more hydrogen atoms) ≤ 5.
2) number of hydrogen bond acceptors (HBAs; nitrogen or oxygen atoms) ≤ 10.
3) molecular weight (M_r) ≤ 500 daltons.
4) octanol-water partition coefficient log $P \leq 5$.

Since the formulation of these rules a number of refinements have been proposed, including a reduction of molecular weight (≤ 300 Da), increased hydrophobicity (log $P \leq 3$), limits on the number of rotatable bonds (≤ 3) and a molecular polar surface area (PSA) of ≤ 140 Å^2. More recently, Bickerton et al., (2012) proposed an empirical measure of drug-likeness called the quantitative estimate of drug-likeness or QED which weights the chosen molecular descriptors (M_r, octanol-water partition coefficient, number of HBDs, number of HBAs, PSA, number of rotatable bonds, number of aromatic rings and absence of unwanted functionalities – see (Brenk, Schipani et al. 2008) to derive a continuum of QED values ranging from zero, where all properties are unfavourable, to one, where all the chosen properties are favourable (Bickerton, Paolini et al. 2012). Despite the increased sophistication of these measures of drug-likeness, it nevertheless remains the case that biological activity must be ultimately determined by some sort of biological assay.

Once bioactive chemical entities have been identified, it is then open to drug designers to modify the structure to improve drug targeting, tissue distribution and solubility to enhance the therapeutic efficacy as drug candidates for clinical trials. An example of this process is Taxol that although being an effective therapeutic in its own

right has given rise to a number of synthetic derivatives including taxotere, ortataxel and docetaxel (Itokawa, Morris-Natschke et al. 2008).

1.4. Flavonoids: Bioactive secondary metabolites from plants

Flavonoids are a large group of plant-specific secondary metabolites. More than 8000 flavonoids have been described (Harborne and Williams 2000; Ververidis, Trantas et al. 2007) and categorised into flavonols, flavones, flavanones, isoflavones, catechins and anthocyanidins (Ross and Kasum 2002). These compounds occur widely in fruits, vegetables, grains, derived beverages (tea, coffee, wine, and beer) and processed foods (syrups, jams, etc.) mainly as flavonoid glycosides (Passamonti, Terdoslavich et al. 2009).

Flavonoids are polyphenolic compounds characterised by a common C15 phenylchromane core, composed of a benzo-γ-pyrone (C6-C3-C6) backbone (Fig. 1.3). Various combinations of multiple hydroxyl, methoxyl and *O*-glycoside substituents on the basic benzo-γ-pyrone can be recruited to form the large number of identified flavonoids (Table 1.1). In the case of the isoflavones, the B ring is connected to the C ring at C3 instead of C2 (Fig. 1.3; Table 1.1).

In plants, flavonoids have roles in signalling, fertility, defence, as antioxidants, pigments and in nodule formation. They are also known to influence plant development by interactions with the cytokinin and auxin families of plant growth substances (Winkel-Shirley 2002; Wasson, Ramsay et al. 2009; Buer, Imin et al. 2010).

The consumption of flavonoids has been associated with a number of health benefits in humans including a decreased risk of heart and cardiovascular disease, improvements of menopausal symptoms and bone resorption (Formica and Regelson 1995; Somekawa, Chiguchi et al. 2001; Hooper, Kroon et al. 2008). Flavonoids are also known to inhibit the proliferation of cancer cells, induce apoptosis, decrease tumour invasiveness, activate the immune response against cancer, modulate the inflammatory cascade and inhibit angiogenesis as part of the treatment strategies for breast, prostate, and colon cancers (Table 1.2) (Neuhouser 2004; Kale, Gawande et al. 2008).

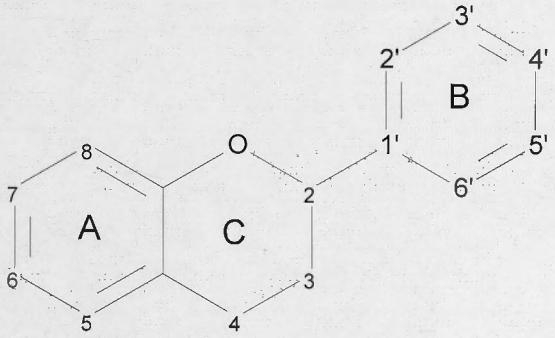
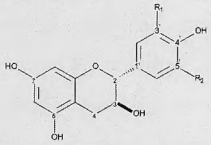
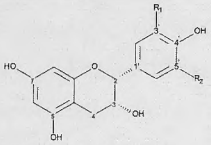
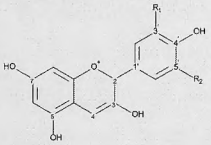
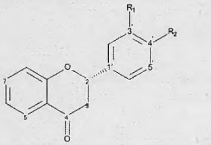
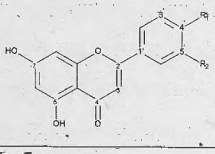
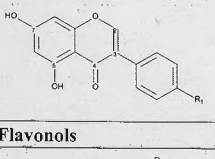
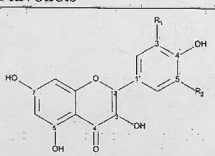


Figure 1.3. Chemical structure of benzo-γ-pyrone backbone.

Flavonoids can be subdivided according to the presence of an oxy group at position 4, a double bond between carbons 2 and 3, or a hydroxyl group in position 3 of the C ring. The only exception to this skeleton is the isoflavones where the B ring is connected via the carbon 3 of the C ring instead of carbon 2 as shown. The hydroxyl groups may also be substituted by a methyl group to yield methoxyls, or a variety of mono- or disaccharides to yield the corresponding glycosides.

Table 1.1. Flavonoids subclasses with some representative compounds and typical food sources.

Subclass	Name	R_1	R_2	Typical rich food sources (Beecher 2003)
Flavanols				
	(+)-Catechin	OH	H	Teas, red grapes and red wines
	(+)-Gallocatechin	OH	OH	
	(-)-Epicatechin	OH	H	
	(-)-Epigallocatechin	OH	OH	
Anthocyanidins				
	Pelargonidin	H	H	Red, purple and blue berries
	Cyanidin	OH	H	
	Delphinidin	OH	OH	
	Peonidin	OCH_3	H	
	Petunidin	OH	OCH_3	
	Malvidin	OCH_3	OCH_3	
Flavanones				
	Naringenin*	H	OH	Citrus fruit and peels
	Eriodictyol*	OH	OH	
	Hesperetin*	OH	OCH_3	
	These compounds are 5,7-dihydroxy-flavanones			
Flavones				
	Apigenin	OH	H	Green leafy spices, e.g., Parsley
	Luteolin	OH	OH	
	Tangeretin *	OCH_3	H	
	Acacetin	OCH_3	H	
	Chrysin	-	-	
	* Tangeretin has OCH_3 at 5, 6, 7 and 8 positions			
Isoflavones				
	Genestein	OH	OH	Soybeans, soy derived foods and legumes
	Daidzein	OH	H	
	Biochanin A	OCH_3	H	
Flavonols				
	Kaempferol	H	H	Nearly ubiquitous in foods
	Quercetin	OH	H	
	Isorhamnetin	OCH_3	H	
	Myricetin	OH	OH	
	Galangin	-	-	
	NB: Galangin has no substituents on the B ring			

Flavonoids can be substituted with one or more sugars at a number of different sites in addition to C7. Adapted from (Passamonti, Terdoslavich et al. 2009).

1.5. Legumes as source of bioactive compounds

1.5.1. Phytoestrogens

Legumes are important dietary components, serving as a source of starch, fibre, protein, lipid and minerals. In addition to their nutritive value, legumes contain significant quantities of phenolic and polyphenolic compounds such as phenolic acids, flavonoids and lignans (Sosulski and Dabrowski 1984; Lin and Lai 2006). The legume attracting much attention recently is the soybean which is a rich source of phytoestrogens including isoflavones and lignans (Setchell 1998; Tham, Gardner et al. 1998). These two classes of phenolic compounds are of relevance to this thesis and their bioactivity in humans will be further discussed in chapters 4 and 5.

Twelve isoflavones have been isolated from soybean including aglycones (genistein, daidzein and glycitine) (Table 1.1), glucosides (genistin, daidzin, glycitin), malonyl glucosides (malonyl daidzin, manolyl genistein, malonyl glycitin), and acetyl glucosides (acetyl daidzin, acetyl genistein, acetyl glycitin) (Wang and Murphy 1994; Wang and Murphy 1994). Six lignans, namely secoisolariciresinol, matairesinol, syringaresinol, lariciresinol, isolariciresinol, and pinoresinol (Fig. 4.17), have also been identified in soybean (Penalvo, Heinonen et al. 2004).

Phytoestrogens have similar chemical structures (2-phenylnaphthalene-type) to oestrogens and have been found to bind to oestrogen receptors (ERβ) (Kuiper, Lemmen et al. 1998). Phytoestrogens are associated with a number of health benefits in humans including reducing levels of chronic diseases such as coronary heart disease and atherosclerosis through the lowering of serum cholesterol and through their anti-oxidative effects. They are also known to have anti-viral, anti-angiogenic, anti-cancer (breast, bowel and prostate) and anti-osteoporosis effects as well as serving to reduce menopausal symptoms (Mazur, Duke et al. 1998; Tham, Gardner et al. 1998; Boue, Wiese et al. 2003; Cornwell, Cohick et al. 2004).

Table 1.2. Clinical effects of flavonoids on animal cells.

Clinical effects	Mechanism of action
Anti-atherosclerotic	Flavonoids have anti-oxidative properties and protect low-density lipoprotein (LDL) from oxygen radicals, which injure the endothelial wall and lower atherosclerotic changes and protect against coronary heart disease (Dewhalley, Rankin et al. 1990; Hertog, Feskens et al. 1993).
Anti-inflammatory	Inhibit the lipoxygenase, cyclooxygenase and 5-lipoxygenase pathways (Moroney, Alcaraz et al. 1988; Ferrandiz, Nair et al. 1990). Inhibiting eicosanoid biosynthesis (Laughton, Evans et al. 1991; Formica and Regelson 1995). Inhibit neutrophil degranulation (Hoult, Moroney et al. 1994; Tordera, Ferrandiz et al. 1994).
Anti-tumour	Inhibition of cell proliferation and angiogenesis (Fotsis, Pepper et al. 1997) by inhibition of protein kinases (Oikawa, Shimamura et al. 1992).
Anti-thrombogenic	Inhibition of platelet aggregation (Osman, Maalej et al. 1998). Inhibition of the activity of cyclooxygenase and lipoxygenase pathways (Alcaraz and Ferrandiz 1987).
Anti-osteoporotic	Prevention of osteoporosis (Hegarty, May et al. 2000).

Adapted from (Nijveldt, van Nood et al. 2001).

1.5.2. Nodulation factors

Part of the agricultural importance of legumes derives from their ability to recruit the *Rhizobium* family of bacteria into symbiotic nodules for the purpose of fixing atmospheric nitrogen. The signalling cascade that controls this process includes the rhizobial secretion of a group of short lipo-chitooligosaccharides (LCOs, also known as nodulation or Nod factors) in response to the legume secreting flavonoids into the rhizosphere. The LCOs enable the rhizobia to facilitate many of the plant's early responses in nodule organogenesis, allowing them to penetrate the root hair at the start of their migration into the root where they form the nitrogen fixing nodules (Fig. 1.4A) (Lerouge, Roche et al. 1990; Cullimore, Ranjeva et al. 2001).

These Nod factors have been found to be active in modulating angiogenesis in *in vitro* and *in vivo* mammalian systems (Patent AU2006/000432) (Djordjevic, Bezos et al. 2013) (Fig. 1.4B and 1.4C). This then led to the idea that further angiogenic modulating compounds may be present in soybean.

1.5.3. Other angiogenesis modulating molecules from soybeans

Preliminary studies by the Parish and Djordjevic laboratories in the Australian Research Council Centre for Integrative Legume Research (CILR) showed, through the use of an *in vitro* angiogenesis bioassay, that soybean xylem sap contained a number of small molecules with either pro- or anti-angiogenic activity in animal cells (Du Fall 2009). These pro- and anti-angiogenic activities were found in the low molecular weight (<1000 Da) fractions from size exclusion chromatography of the soybean sap, however, insufficient material was isolated for any structural elucidation to be attempted. My thesis builds upon these initial findings.

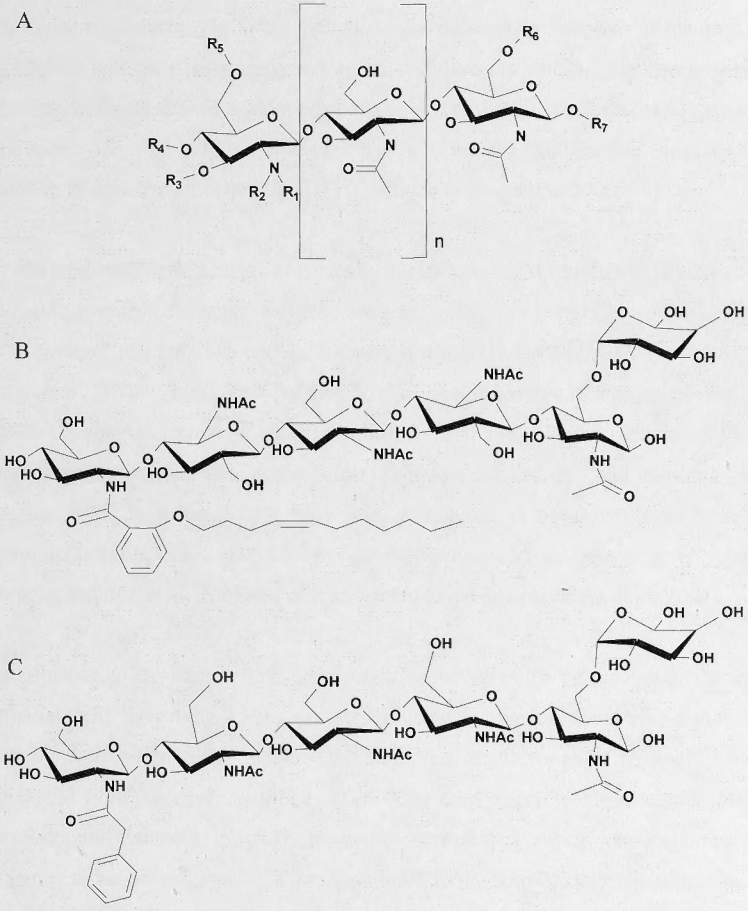


Figure 1.4. Chemical structures of angiogenesis modulating Nod factors.

Generic structure of Nod factors (chitin parameter backbone), n=1-3, R_1=H, Me, R_2= C16:2, C16:3, C18-C26(ω-1)OH, C18:1, C18:3, C18:4, C20:3, C20:4, $R_{3,4,5}$= Ac(O-6), Cb, Cb(O-3), Cb(O-4), Cb(O-6), H, R_6= S, H, Ac, AcFuc, MeFuc, AcMeFuc, R_7= H, glycerol (A), Anti-angiogenic compound (m-Benzamide, C18:1, Fucosyl) (B), Pro-angiogenic compound (Phenyl acetyl, Fucosyl) (C). Me, methyl; Ac, acetyl, Fuc, fucosyl; Cb, carbamoyl; S; sulphate ester. Reproduced from (Denarie, Debelle et al. 1996).

1.6. Biology of angiogenesis

Blood vessels transport blood into growing and mature tissues, providing nutrients, oxygen, hormone delivery and the removal of waste materials (Folkman, Bach et al. 1971; Clapp, Thebault et al. 2009). This vascular network is essential for the growth of both healthy and neoplastic tissues (Papetti and Herman 2002) and the process by which mature blood vessels are remodelled to form new capillary networks is known as angiogenesis. In simple terms, angiogenesis involves the proliferation, migration and maturation of endothelial cells (Fig. 1.5) (Griffioen and Molema 2000).

In adult tissues, angiogenesis is normally under very tight regulation (Ejaz and Lim 2005) and is inhibited, except for processes including the female reproductive cycle, wound healing, placentation and in fracture healing (Folkman 2007; Samaranayake, Maatta et al. 2010; Chung and Ferrara 2011). Angiogenesis is also associated with various disease and pathological conditions including inflammation, infection, retinopathies, atherosclerosis, peptic ulcers, rheumatoid arthritis and tumourogenesis (Carmeliet 2003; Folkman 2007). However, insufficient angiogenesis leads to various ischemic conditions such as ischemic heart disease and peripheral artery disease (Tammela, Enholm et al. 2005) as well as hypertension (Sane, Anton et al. 2004).

As illustrated in Figure 1.5, in a healthy adult body (non-angiogenic state), endothelial cells have long half-lives and are protected by low level stimulation from angiogenic factors including vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) and fibroblast growth factors (FGFs), to help maintain blood vessel integrity (Carmeliet and Jain 2011). Angiogenesis is initiated when vessels receive an angiogenic signal from growth factors such as VEGF, Ang-2, basic fibroblast growth factor (bFGF/FGF-2) or platelet-derived growth factor (PDGF) induced by angiogenic stimuli (hypoxia, inflammation or tumour cells) (Collinson and Donnelly 2004; Pandya, Dhalla et al. 2006). In the case of Ang-2, it will compete with Ang-1 in binding to endothelial Tie-2 receptors on the endothelial cells, a process which results in the release of matrix metalloproteinases (MMPs) into the extracellular matrix (ECM). In tumourogenesis, MMPs are also secreted from tumour cells along with VEGF, activating endothelial cells and facilitating the formation of endothelial cell sprouts which can then develop into new capillaries (Carmeliet and Jain 2011).

MMPs also mobilise pro-angiogenic proteins from the stroma by proteolytic degradation of the basement membrane, as well as removing local angiogenesis inhibitors by, for example, cleaving endostatin from collagen XVIII in the vessel wall and angiostatin from circulating plasminogen (Folkman 2007). Integrins and cadherins are cell adhesion molecules which facilitate cell-ECM and cell-cell binding, respectively, and are required to maintain the viability of the endothelial cells and their responsiveness towards regulatory signals. In angiogenesis, certain pro-angiogenic proteins can up-regulate endothelial integrins and in response to induced integrin signalling, endothelial cells migrate into the ECM (Liekens, De Clercq et al. 2001; Otrock, Mahfouz et al. 2007). In the meantime, the proteolytic action of MMPs on the ECM liberates more angiogenic molecules, such as FGF, Ang-2 and VEGF, which bind to the glycosaminoglycan heparan sulphate in the ECM. To form a tube from the proliferating endothelial cells, one cell, known as the tip cell, is selected via signalling pathways induced by receptors such as VEGF receptors and neuropilins (NRPs) (Cross and Claesson-Welsh 2001; Carmeliet and Jain 2011). Not all new endothelial cells are derived from proliferating endothelial cells at the site of angiogenesis as some will be provided by bone marrow endothelial cells. In the later stages of angiogenesis, smooth muscle cell pericyte and fibroblast recruitment leads to the formation of mature thick walled vessels (Fig. 1.5) (Folkman 2007).

As mentioned above, some growth factors are delivered directly by tumour cells to the local endothelium. However, it has also been found that some of the angiogenesis regulators including both pro- and anti-angiogenic proteins, are scavenged by platelets and stored in different sets of alpha granules (Fig. 1.5) (Folkman 2007).

1.7. Regulation of angiogenesis

Angiogenesis is a complex and highly controlled process of interaction of various endogenous angiogenic stimulators and inhibitors (Table 1.3). Growth factors and cytokine-related peptide signalling molecules are the key regulators of angiogenesis and are synthesised by both normal and malignant cells. VEGFs (Ferrara 2002) and FGFs are the best characterised of the pro-angiogenic regulators (Turner and Grose 2010; Tenhagen, van Diest et al. 2012).

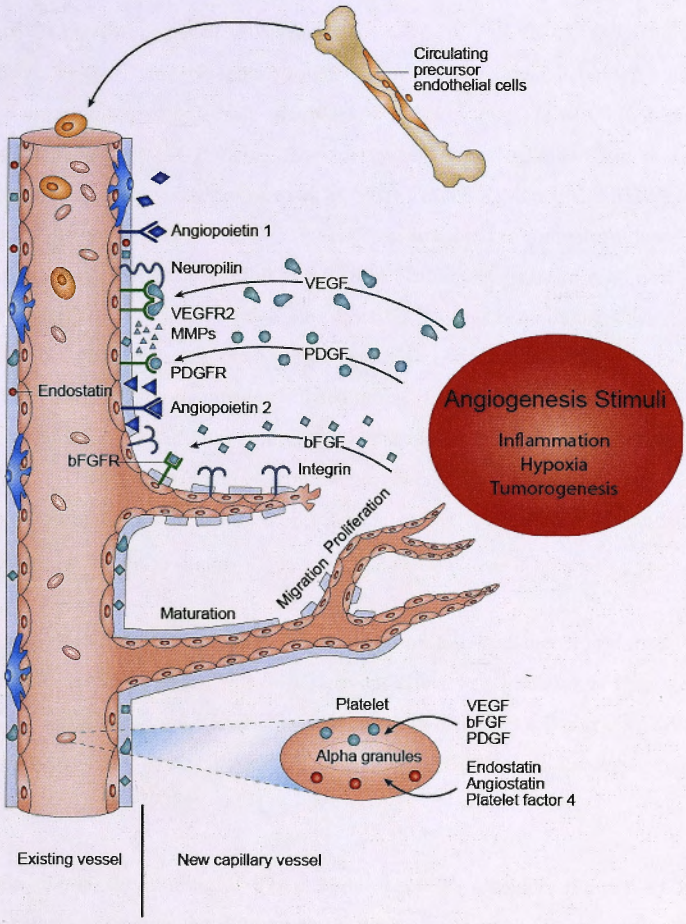


Figure 1.5. Overview of the molecular mechanisms and key cellular steps involved in angiogenesis.

The process of formation of new capillaries from pre-existing vessels involves the major cellular steps of proliferation, migration and maturation of endothelial cells (EC). In response to angiogenic stimuli, induced angiogenic factors interact with receptors on endothelial cells and trigger the formation of new blood vessels. Adapted from (Folkman 2007).

1.7.1. Vascular endothelial growth factor (VEGF)

The VEGF family consist of seven members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placenta growth factor (PlGF) 1 and 2, which selectively bind to 3 types of tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1 or KDR) and VEGFR-3 (Flt-4) (Hoeben, Landuyt et al. 2004; Roy, Bhardwaj et al. 2006). Neuropilins (semaphoring receptors), such as NRP-1 and NRP-2 are VEGF co-receptors and enhance the activity of VEGFR-2 but also are involved in axonal guidance during neural development (Chen, Chedotal et al. 1997; Tammela, Enholm et al. 2005; Roy, Bhardwaj et al. 2006). VEGF-A (also known as VEGF) has been identified as the main component that stimulates angiogenesis in health and disease (embryogenesis, wound healing and during tumour growth) by binding to VEGFR-1 and VEGFR-2 on endothelial cells (Roy, Bhardwaj et al. 2006; Carmeliet and Jain 2011) whereas VEGF-C and VEGF-D can also induce lymphangiogenesis by binding to VEGFR-3 on lymphatic endothelium (Fig. 1.6) (Ferrara 2001; Tammela, Enholm et al. 2005; Alitalo 2011).

In humans, VEGF is a secreted 34-42 kDa glycoprotein with seven isoforms, VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{148}, VEGF-A_{165} (most effective stimulator of angiogenesis), VEGF-A_{183}, VEGF-A_{189} and VEGF-A_{206} as a result of alternate splicing of mRNA from a single, 8-exon, VEGF gene (Ferrara 2001; Ferrara 2004; Hoeben, Landuyt et al. 2004; Nowak, Woolard et al. 2008).

In angiogenesis, the binding of VEGF to its receptors increases the permeability of the endothelium including the formation of intercellular gaps (Bates, Hillman et al. 2002). VEGF also induces proliferation, sprouting, migration and tube formation of endothelial cells (Ferrara, Gerber et al. 2003; Otrock, Mahfouz et al. 2007). The main signalling pathways involved in VEGF-mediated angiogenesis are phosphoinositide 3-kinase/anti-apoptotic kinase (PI3K/Akt), mitogen activated protein kinase (MAPK) and protein kinase C (PKC) (Cross and Claesson-Welsh 2001; Roy, Bhardwaj et al. 2006; Otrock, Mahfouz et al. 2007). Several VEGF blockers have been approved for anti-angiogenic therapy in cancer and eye disease (Jain, Duda et al. 2006; Ferrara 2009). The US Food and Drug Administration (FDA) has approved the use of the VEGF-antibody bevacizumab (Avastin) for metastatic colorectal cancer, metastatic non-small cell lung cancer, metastatic breast cancer and metastatic renal cell carcinoma (RCC).

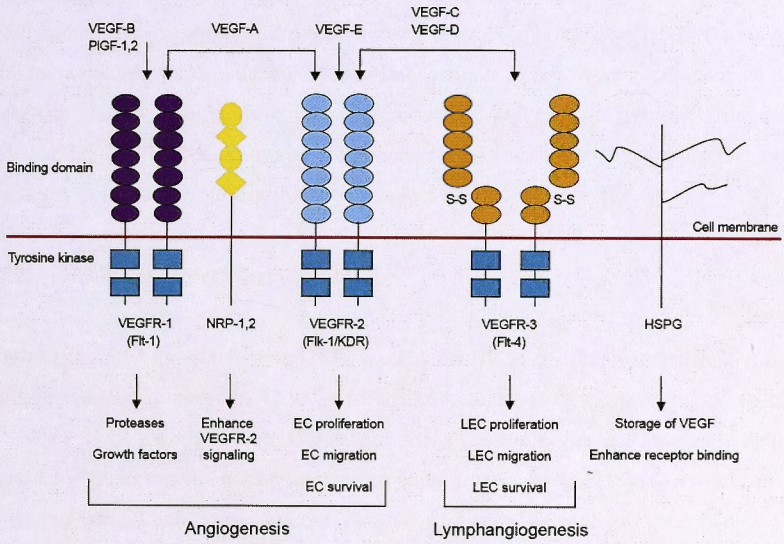


Figure 1.6. The VEGF family ligands and their receptors.

VEGFR-1 (Flt-1), VEGFR-2 (KDR Flk-1), and VEGFR-3 (Flt-4) are signalling tyrosine kinase receptors with seven immunoglobulin-like structures in the extracellular domain, a single trans-membrane region, and a consensus tyrosine kinase domain interrupted by a kinase insert domain. The neuropilin-1 and neuropilin-2 receptors (NRP-1,2) do not have kinase activity but enhanceVEGFR-2 signalling. The heparan sulphate proteoglycan (HSPG) not only enhances VEGFR binding but also serves as a reserve of VEGF. VEGF-A, VEGF-B and PlGF-1,2 bind VEGFR-1 and VEGFR-2 on the blood vascular endothelium and VEGF-C and VEGF-D primarily bind VEGFR-3 on lymphatic endothelium inducing angiogenesis and lymphangiogenesis, respectively. Adapted from (Hoeben, Landuyt et al. 2004; Tammela, Enholm et al. 2005).

Also, sorafenib (Nexavar), sunitinib (Sutent) and pazopanib (Votrient), which block the VEGF signalling pathway, have been approved for metastatic RCC (Carmeliet and Jain 2011). Moreover, angiogenic therapeutic strategies using VEGF might be beneficial and may lead to increased neovascularization in some cardiovascular disorders (Khurana, Simons et al. 2005).

However, the clinical use of VEGF inhibitors in cancer patients has been shown to be challenging. For example, the use of bevacizumab is approved only when combined with cytotoxic or cytokine therapy (Carmeliet and Jain 2011). Also, many patients with metastatic disease may show rejection or resistance to VEGF inhibitors (Bergers and Hanahan 2008). Therefore, more comprehensive studies need to be conducted in order to achieve promising results in this therapeutic area.

1.7.2. Fibroblast growth factors (FGFs)

The fibroblast growth factors (FGFs) are a family of 23 secreted and cell surface proteins with a size range of 17 to 34 kDa (Ornitz and Itoh 2001; Javerzat, Auguste et al. 2002). FGFs have a high affinity for the glycosaminoglycan, heparan sulphate, carried by glycoproteins called heparan sulphate proteoglycan (HSPG) located on the surface of certain cell types, especially endothelial cells, as well as in the ECM. This association assists the binding of FGFs to four highly conserved transmembrane bound tyrosine kinase FGF receptors (FGFR). This results in a phosphorylation cascade of target proteins and activation of several parallel signalling pathways, including PLCγ, STATs, AKT and MAPK, which alters the pattern of gene expression with resultant stimulation of angiogenesis (Fig. 1.7) (Powers, McLeskey et al. 2000; Cross and Claesson-Welsh 2001; Turner and Grose 2010; Tenhagen, van Diest et al. 2012).

FGFs are involved, firstly, in the early invasive phase of angiogenesis during embryonic development, tissue repair, wound healing and tumour angiogenesis, which involve degradation of basal lamina, migration and cell proliferation. They are also involved in the later phase of vessel maturation, regulating endothelial cell morphogenesis and vessel maturation (Eswarakumar, Lax et al. 2005; Presta, Dell'Era et al. 2005).

Table. 1.3. Endogenous angiogenesis stimulators and inhibitors. Adapted from (Li, Li et al. 2012).

Stimulators	Inhibitors
β-Estradiol	2-Methoxyestradiol
Adenosine	Angiopoietin-2 (in the absence of VEGF)
Adrenomedullin	Angiostatin (plasmin fragment)
Aminopeptidase N/CD13	Anti-angiogenic anti-thrombin III
Angiogenin	Arresten (fragment of collagen IV α1 chain)
Angiopoietin-1 (Ang-1)	Canstatin (fragment of the α2 chain of type IV collagen)
Endogenous opioids (Endomorphin-1, endomorphin-2 and deltorphin I)	Chondromodulin I
Endothelial cell stimulating angiogenesis factor (ESAF)	Connective tissue growth factor (CTGF)
Erythropoietin (EPO)	Decorin
Fibroblast growth factors (FGFs); 23 members	Endorepellin (Endorepellin, a C-terminal fragment of the vascular basement membrane proteoglycan perlecan)
Follistatin	Endostatin (collagen XVIII fragment)
Granulocyte-colony-stimulating factor (G-CSF)	Fibronectin fragment
Heme oxygenase (HO-1)	Histidine-rich glycoprotein (HRR domain)
Heparanase	Interferon-α,β,γ (IFN-α,β,γ)
Hepatocyte growth factor (HGF)	Interferon-inducible protein-10 (IP-10)
Hepatocyte growth factor/scatter factor (HGF/SF)	Interleukin-4,-10, -12,-18 (IL-4, IL-10, IL-12, IL-18)
Histidine-rich glycoprotein (HRG)	Kringle 5
Hyaluronan oligosaccharides	Maspin
Hypoxia-induced factor-1 (HIF-1)	Metastatin
Interleukin-3, -8 (IL-3, IL-8)	METH-1
Intermedin	METH-2
Leptin	Osteopontin cleavage product
Macrophage chemoattractant protein-1 (MCP-1)	PEX
Matrix metalloproteases (MMPs)	Pigment epithelium derived factor (PEDF)
Midkine	Plasminogen activator inhibitor-1 (PAI-1)
Neuregulin	Platelet factor-4
Nitric oxide synthase (NOS)	Prolactin (16 kDa fragment)
Osteogenic protein-1	Proliferin-related protein
Oxytocin (OT)	Prothrombin kringle 2
Placental growth factor (PlGF)	Restin
Platelet-derived endothelial-cell growth factor (PD-ECGF)	Soluble fms-like tyrosine kinase-1 (S-Flt-1)
Platelet-derived growth factor (PDGF)	SPARC cleavage product
Pleiotrophin	Tetrahydrocortisol-S
Progranulin	Thrombospondin-1,2 (TSP-1, TSP-2)
Proliferin	Tissue inhibitors of matrix metalloproteinases (TIMPs)
Stromal-derived factor-1α (SDF-1α)	Transforming growth factor-β (TGF-β) (activated form)
Thyroid hormone	Troponin-1
Transforming growth factor-α (TGF-α)	Tumstatin (fragment of the α3 chain of type IV collagen)
Transforming growth factor-β (TGF-β)	Vascular endothelial growth inhibitor (VEGI)
Tumour necrosis factor-α (TNF-α)	Vasostatin
Urotensin-II (U-II)	
Vascular endothelial growth factors (VEGFs); 7 members	

Basic FGF (bFGF, FGF-2) is the most potent angiogenesis inducer in the FGF family (Ferrara, Chen et al. 1998; Bouis, Kusumanto et al. 2006). It is ubiquitously expressed in cells of mesodermal and neuroectodermal origin at low and high concentrations, respectively, as well as in a variety of tumour cells (Seghezzi, Patel et al. 1998). FGF-2 is a potent mitogen for different cell types, including vascular endothelial cells, fibroblasts, smooth muscle cells and glial cells (Nugent and Iozzo 2000; Liekens, De Clercq et al. 2001) and has been reported to induce proliferation, migration, proteinase production, and expression of specific integrins (e.g., $\alpha V\beta 3$) on cultured endothelial cells (Moscatelli, Presta et al. 1986; Klein, Bikfalvi et al. 1996; Presta, Dell'Era et al. 2005).

It has been shown that increased levels of bFGF expression as measured in the serum (approximately 10%) (Folkman 1993) is associated with progression or tumour recurrence of lung, prostate, breast, bladder and esophageal cancers (Faridi, Rudlowski et al. 2002; Polnaszek, Kwabi-Addo et al. 2003; Barclay, Li et al. 2005; Shariat, Youssef et al. 2010). Also, bFGF has been tested in human trials for neovascularisation for conditions such as myocardial ischemia and skeletal muscle ischemia in order to induce cardiac repair (Zhao, Zhao et al. 2011). However, it failed in a phase II clinical trial to show significant efficacy in treating coronary artery disease but in another trial it showed a significant therapeutic effect in treating peripheral artery disease, although the benefit was not regarded as satisfactory at the dose tested (Aviles, Annex et al. 2003; Zhao, Zhao et al. 2011).

Based on the current knowledge of the bFGF and FGF-FGFR signalling pathways, FGF-2 may prove to be a valuable marker for cancer progression and a target for anti-angiogenic therapies (Turner and Grose 2010) as well as therapeutic angiogenesis (pro-angiogenesis) (Aviles, Annex et al. 2003).

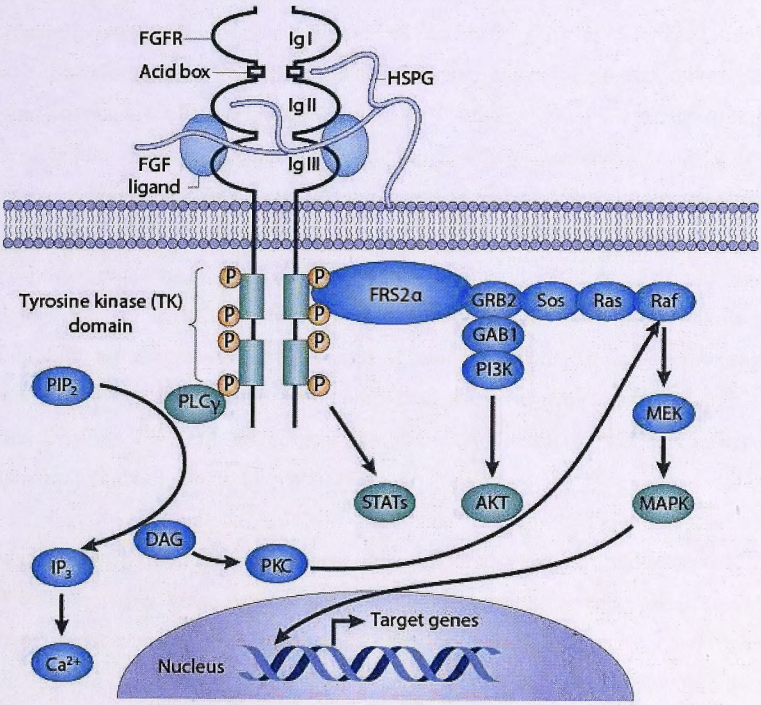


Figure 1.7. FGF-FGFR structure and downstream signalling.

The extracellular domain of FGFR consists of three ligand binding (Ig) sites. Binding of FGF is stabilised by the heparan sulphate proteoglycan (HSPG) chain. Once, FGF-FGFR binding occurs, the receptor dimerises and the kinase domains transphosphorylate each other. This leads to the docking of adaptor proteins and activation of four key downstream pathways: PLCγ, STATs, AKT and MAPK. FGF, fibroblast growth factor; HSPG, heparan sulphate proteoglycans; TK, tyrosine kinase domain; STAT, signal transducer and activator of transcription; FRS2α, fibroblast growth factor receptor substrate 2a; GRB2, growth factor receptor-bound protein 2; GAB1, GRB2-associated binding protein 1; PI3K, phosphoinositide-3 kinase; AKT, v-akt murine thymoma viral oncogene; SOS, son of sevenless; RAS, rat sarcoma; MAPK, mitogen-activated protein kinase; PLCγ, phospholipase Cγ; PIP2, phosphatidylinositol 4,5-biphosphate; IP3, inositol 1,4,5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C. Adapted from (Turner and Grose 2010).

1.8. Significance of angiogenesis as a therapeutic target

The disruption of the regulatory mechanisms controlling angiogenesis can have a significant impact on health as it underlies the pathology of diseases characterised by uncontrolled blood vessel formation, including cancer, psoriasis, arthritis, retinopathies, obesity, asthma, and atherosclerosis (Table 1.4) (Carmeliet 2003; Carmeliet 2005). In 1971, Folkman first introduced the concept of "anti-angiogenesis" as a potential anticancer strategy. He suggested that angiogenesis can be a target for the treatment of solid tumours since malignant tumours cannot grow beyond 2-3 mm^3 without recruiting new capillary blood vessels to supply the nutrients required for further cell proliferation (Folkman, Bach et al. 1971). Therefore anti-angiogenic agents can effectively be used for inhibiting the progression or recurrence of tumour growth. To date, approximately 50 anti-angiogenic agents have been discovered, some of which have already been approved by the US FDA for clinical use and scores of others that are undergoing clinical trials (Table 1.5) (Li, Li et al. 2012).

In addition, insufficient angiogenesis and abnormal vessel regression can lead to heart and brain ischemia, neurodegeneration, hypertension, osteoporosis, respiratory distress, preeclampsia, endometriosis, postpartum cardiomyopathy, and ovarian hyperstimulation syndrome (Table 1.4) (Carmeliet 2003; Carmeliet 2005). So far, clinical studies of pro-angiogenic therapeutic strategies have involved the administration of growth factors such as VEGF and FGF to stimulate angiogenesis. As mentioned in sections 1.7.1 and 1.7.2, despite pre-clinical *in vivo* trials showing promise, clinical trials testing VEGF and FGF-2 have failed at the phase II or III stages (Stewart, Kutryk et al. 2009; Zhao, Zhao et al. 2011). Therefore more studies need to be conducted to discover more effective pro-angiogenic agents.

1.9. Natural small molecules as exogenous angiogenesis modulators

Angiogenic modulators are agents that can interact with the regulatory pathways controlling angiogenesis to either enhance or inhibit angiogenesis (Cristofanilli, Charnsangavej et al. 2002). Due to the critical role of angiogenesis in health and disease, the identification of strategic drugs that regulate angiogenesis has been a challenge for clinical investigators. Although, the majority of angiogenic regulators are typically endogenous proteins such as VEGF and FGF, as described previously, the

research for small exogenous molecules that are capable of either promoting or inhibiting angiogenesis may offer rational targets for drug development.

There are several examples of traditional medicines affecting the process of angiogenesis and these have then been studied to identify the active constituent. For example, silibinin (also known as silybin), a flavonolignan, is the active constituent of Silymarin, a standardised extract derived from the milk thistle plant (*Silybum marianum*) (Fig. 1.8A). Silymarin has been used for more than 2000 years as a natural remedy for treating hepatitis and cirrhosis and to protect the liver from toxic substances (Ramasamy and Agarwal 2008). Silymarin has also been extensively studied in both *in vivo* and *in vitro* experiments for its anti-angiogenic effect against various cancers including colon, breast and prostate cancers (Yang, Lin et al. 2003; Davis-Searles, Nakanishi et al. 2005).

It has been shown that some of its anti-angiogenic effect has been associated with inhibition of VEGF secretion, down regulation of VEGFR-3, up-regulation of angiopoietin-2 (Zi, Feyes et al. 1998; Jiang, Agarwal et al. 2000; Agarwal, Agarwal et al. 2006; Singh, Deep et al. 2006) and down-regulation of MMP-2 (Lah, Cui et al. 2007).

Another traditional oriental medicinal plant *Cinnamomum cassia* has been used to improve blood circulation (Choi, Baek et al. 2009). It has also been shown to affect bone formation in osteoblastic cells and consequently contributes to the prevention of osteoporosis and inflammatory bone diseases (Lee and Choi 2006). The active constituent was identified as cinnamic acid and this has now been used as a novel pro-angiogenic compound which, in *in vitro* and *in vivo* studies was found to up-regulate VEGF and the VEGF receptors (VEGFR-2/Flk-1/KDR) (Fig. 1.8B) (Choi, Baek et al. 2009).

Tables 1.6 and 1.7 show more examples of angiogenesis modulating small molecules derived from natural sources. However, the majority of the low molecular weight exogenous molecules are inhibitors of angiogenesis. Therefore, this thesis is focused on the identification of novel pro-angiogenic molecules from the soybean plant which already has a well-established reputation for providing human health benefits.

Table 1.4. Examples of diseases where targeting angiogenesis has therapeutic application.

Disease	Treatment strategy	Therapeutic effect
Cancer	Inhibition of blood vessel growth to solid tumours	Anti-angiogenesis
Infectious disease	Inhibiting angiogenesis stimulated by angiogenic promoting genes of pathogens	Anti-angiogenesis
Obesity	Inhibiting angiogenesis induced by a fatty diet	Anti-angiogenesis
Psoriasis	Preventing dermal microvascular expansion that supports excessive skin production	Anti-angiogenesis
Diabetic retinopathy	Inhibiting abnormal blood vessel growth that contributes to diabetes related vision loss	Anti-angiogenesis
Asthma	Inhibiting angiogenesis that could contribute to abnormal tissue growth	Anti-angiogenesis
Arthritis	Inhibiting angiogenesis to prevent tissue lining the joints from invading and destroying cartilage and bone leading to arthritis	Anti-angiogenesis
Stroke	Stimulation of angiogenesis in the brain, restoring oxygen supply, could increase survival chance and preventing stroke if administered following early warning signs	Pro-angiogenesis
Hypertension	Treat by reversing the effects of microvessel rarefaction due to impaired angiogenesis	Pro-angiogenesis
Diabetes	Restoring peripheral bloody supply to ischemic limbs	Pro-angiogenesis
Osteoporosis	Preventing the age dependent decline of VEGF stimulated angiogenesis	Pro-angiogenesis

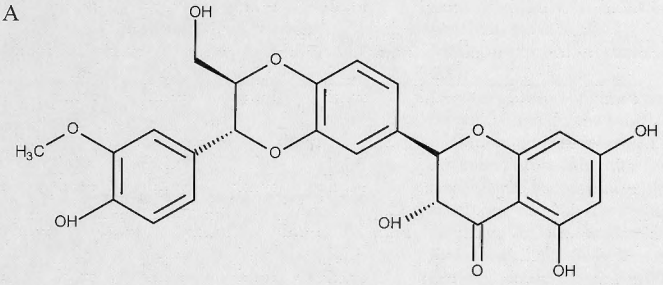
Adapted from (Carmeliet 2003).

Table 1.5. Examples of FDA approved anti-angiogenic agents.

Agent	Cancer type	Target
Bevacizumab (Avastin)	Colon, Lung, Breast, Brain, Kidney	VEGF
Cetuximab (Erbitux)	Colon, Head and Neck	EGFR
Endostatin*	Lung	bFGF/VEGF
Erlotinib (Tarceva)	Lung, Pancreatic	EGFR
Everolimus	Kidney, Pancreatic, Brain	mTOR
Gefitinib (Iressa)	Lung, breast	EGFR
Imiquimod	Actinic keratosis, Basal cell carcinoma	Activate the immune system mostly via TLR7
Interferon α (IFN-α)	Melanoma, Kaposi's sarcoma	
Lenalidomide	Myleodysplastic syndrome, Multiple myeloma	Direct anti-tumour effect Inhibition of the microenvironment support for tumour cells Immunomodulatory role
Pazopanib (Votrient)	Kidney	VEGFR1, 2, 3
Pegaptanib (Macugen)	Age-related macular degeneration	VEGF
Sorafenib	Kidney, Liver	VEGFR, PDGFR, Raf kinases
Sunitinib	Kidney, GIST, Pancreatic	RTK
Temsirolimus (Torisel)	Kidney, Lymphoma	mTOR, VEGF
Thalidomide	Multiple myeloma	bFGF
Vandetanib	Thyroid	VEGFR

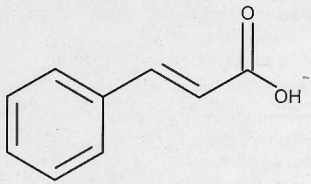
bFGF, basic fibroblast growth factor; EGFR, epidermal growth factor receptor; mTOR, mammalian target of rapamycin; PDGFR, platelet-derived growth factor receptor; RTKs, receptor tyrosine kinases; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; TLR7, toll-like receptor 7. Adapted from (Li, Li et al. 2012).
*Endostatin is approved by the State FDA in China.

A



Silibinin

B



Cinnamic acid

Figure 1.8. Chemical structures of silibinin (*Silybum marianum*), the anti-angiogenic constituent of Silymarin (A) and cinnamic acid, the pro-angiogenic compound derived from *Cinnamomum cassia* (B).

Table 1.6. Examples of exogenous anti-angiogenic small molecules from natural products.

Compounds	Natural source	Mechanism of action
Anthocyanins	Berries, grapes, red wine	Suppression of VEGF and HIF-1α expression (Lu, Li et al. 2006; Wang, Dombkowski et al. 2011). MMP-10 expression (Stoner, Chen et al. 2006).
Curcumin	*Curcuma longa,* turmeric spice	Down-regulation of gene transcripts for VEGF, bFGF, and MMP-2; COX-2 inhibition; up-regulation of TIMP; disruption of vascular tube formation; and inhibition of endothelial cell motility by interfering with the Ras-mediated JNK pathway (Thaloor, Singh et al. 1998; Mohan, Sivak et al. 2000; Shin, Kim et al. 2001; Gururaj, Belakavadi et al. 2002; Shao, Shen et al. 2002).
Ellagitannins	Pomegranate, strawberries, blackberries, raspberries, muscadine grapes, walnuts and pecans	Suppression of secretion of VEGF and HIF-1α (Sartippour, Seeram et al. 2008).
Epigallocatechin-3-gallate (EGCG)	Green tea	Inhibition of endothelial cell proliferation stimulated by bFGF (Cao and Cao 1999). Inhibition of VEGF expression (Liao, Yang et al. 2004). Inhibition of MMP-2 and MMP-9 (Garbisa, Biggin et al. 1999; Garbisa, Sartor et al. 2001).
Genistein	Soybean	Inhibition of bFGF- and VEGF-driven endothelial cell proliferation, migration, and tube formation; inhibition of extracellular matrix degradation by suppression of bFGF-induced endothelial production of PA and PAI; and suppression of RTK activity for VEGF, EGF and PDGF (Akiyama, Ishida et al. 1987; Fotsis, Pepper et al. 1993).
Lycopene	Fruits with bright red colour such as tomato, watermelon and papaya	Suppresses signalling by PDGF and Platelet Activation Factor (Wu, Chiang et al. 2007).
Menaquinone (vitamin K2, fat soluble)	Bacteria in fermented dairy products, including cheese and yogurt, soy and also dark meat	Inducing apoptosis (Shibayama-Imazu, Aiuchi et al. 2008).
Omega-3, Polyunsaturated fatty acids (PUFAs)	Cold water oily fish such as salmon, herring, mackerel, anchovies, sardines and trout	Down-regulating Ang-2 (Szymczak, Murray et al. 2008). Suppression of Akt/m-TOR signalling pathway (Friedrichs, Ruparel et al. 2011).
Quercetin	Numerous types of fruits and vegetables	Inhibition of MMP-2 and MMP-9 secretion (Davis, Emenaker et al. 2010).

bFGF, basic fibroblast growth factor; HIF-1α, Hypoxia-inducible factor 1-α; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor; JNK, c-Jun N-terminal kinase; PAI, plasminogen activator inhibitor; PA, plasminogen activator receptor; Ang-2, angiopoietin-2; COX-2, cyclooxygenase-II; MMP-2,-9, matrix metalloproteinase-2,-9,-10; TIMPs, tissue inhibitors of matrix metalloproteinase; EGF, endothelial growth factor. Adapted from (Li, Li et al. 2012).

Table 1.7. Examples of exogenous pro-angiogenic small molecules from natural products.

Compounds	Natural source	Mechanism of action
Cinnamic acid	*Cinnamomun cassia*	Up-regulation of VEGF and Flk-1/KDR (VEGF receptor-2) (Choi, Baek et al. 2009).
Dibenzoylmethane (DBM)	Licorice plants	Increasing HIF-1α protein and VEGF secretion (Mabjeesh, Willard et al. 2003).
Nicotine	Plants of the Solanaceae family, such as tobacco (*Nicotiana tabacum*) and the coca plant (*Erythroxylum coca*)	Stimulation of nicotinic acetylcholinergic receptors (particularly); inducing the expression of bFGF, PDGF and VEGF; up-regulation of Flk-1/KDR; increasing the recruitment of endothelial progenitor cells (EPC) into blood vessels (Mousa and Mousa 2006; Costa and Soares 2009; Egleton, Brown et al. 2009).
Resveratrol	Red grapes	Stimulation of Trx-1, HO-1 and VEGF synthesis (Kaga, Zhan et al. 2005). Increasing expression of NFkB and Sp-1 (Fukuda, Kaga et al. 2006). Increasing VEGF and Flk-1 expression (Wallerath, Deckert et al. 2002).
Sokotrasterol Sulphate	Marine sponges	Up-regulation of bFGF (Murphy, Larrivee et al. 2006).

bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor ; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; Trx-1, Thioredoxin-1; HIF-1α, Hypoxia-inducible factor 1-α; HO-1, heme oxygenase-1; NFkB, nuclear factor kappa B; Sp1, specificity protein 1.

1.10. Angiogenesis bioassays

The key to successful natural product drug discovery is the availability of an appropriate assay for the desired bioactivity (New, Miller-Martini et al. 2003; Koehn and Carter 2005). Angiogenesis bioassays are an essential tool not only for screening for angiogenic activity but also for studying the mechanism of action of active compounds (Donovan, Brown et al. 2001). Angiogenesis assays can be performed either in *in vivo* or *in vitro* settings. As reviewed by Jain et al., 1997 and Auerbach et al., 2003, numerous *in vivo* assays have been developed in recent years including the chick chorioallantoic membrane (CAM) assay, the Matrigel plug assay, and a group of assays using implants of sponges containing test cells or compounds in animal bodies (Jain, Schlenger et al. 1997; Auerbach, Lewis et al. 2003).

Although *in vivo* assays give a direct and more comprehensive insight into how the test compounds might affect the angiogenic process, there are a number of disadvantages associated with their use including being expensive, time consuming and sometimes a source of ethical problems (Donovan, Brown et al. 2001).

In vitro assays provide critical information as a first step of angiogenic compound validation as they tend to be less expensive, less time consuming, more reproducible, easier to monitor and have fewer ethical problems. As the aim of this PhD thesis project is to screen a large number of compounds and chromatographic fractions for angiogenic activity, an *in vitro* angiogenesis assay was employed (Jain, Schlenger et al. 1997; Donovan, Brown et al. 2001; Auerbach, Lewis et al. 2003). *In vivo* assays are required in the later steps of drug development but they have not been used in the research described in this thesis and, therefore, will not be discussed further.

The *in vitro* angiogenesis assays involve using either a single cell type (cellular) or organ culture (organotypic) assays (Goodwin 2007). *In vitro* cellular assays are often used to study one particular step in angiogenesis such as proliferation, migration and differentiation of endothelial cells. These assays can portray a specific part of the angiogenic cascade being affected by the test compounds (Bootle-Wilbraham, Tazzyman et al. 2000; Donovan, Brown et al. 2001). Therefore, these assays can be employed to study the cellular mode of action of angiogenically active compounds.

The organotypic assays allow the effect of a test agent on multiple cell types in the angiogenesis environment to be analysed and include mouse metatarsal, rat and mouse aortic ring and chick aortic arch assays. Both mouse metatarsal (using embryonic mouse bones) and chick aorta arch (using embryonic aorta arches) assays have the disadvantage of the cells themselves undergoing rapid cell proliferation and angiogenesis which make them less ideal bioassays for screening for pro-angiogenic compounds (Auerbach, Lewis et al. 2003; Goodwin 2007).

1.10.1. *In vitro* rat aorta ring assay

The *in vitro* rat aorta ring assay is a 7-14 day assay in which the thoracic aorta is cut into approximately 1 mm lengths and embedded into a matrix-containing environment such as Matrigel, fibrin or collagen gels. Among the available *in vitro* angiogenesis assays, the rat aorta bioassay (Nicosia and Ottinetti 1990) represents a more ideal *in vitro* model in that it bridges the gap between the *in vivo* and *in vitro* models. Firstly, the wounding that occurs during the cutting of the aorta is sufficient to induce angiogenesis and hence exogenous factors do not need to be added. Secondly, it reproduces more accurately the environment in which angiogenesis takes place *in vivo* than alternative procedures using preselected endothelial cells and their subsequent *in vitro* passaging. Thirdly, it not only includes endothelial cells but also the surrounding cells which are also involved in angiogenesis. Fourthly, the pro-angiogenic factors are best tested in the assays where baseline angiogenesis is relatively low, as with the rat aortic ring assay, rather than in *in vitro* assays using embryonic organs (Auerbach, Lewis et al. 2003; Goodwin 2007).

On the other hand, since the *in vivo* angiogenesis mainly occurs at the microvascular level, the use of aorta may be a disadvantage (Auerbach, Lewis et al. 2003; Goodwin 2007). However, since the *in vitro* rat aorta assay was instrumental in the identification of PI-88 (also known as Muparfostat) (Parish, Freeman et al. 1999) (Fig. 1.9), an anti-angiogenesis compound that has progressed to advanced (phase III) clinical trials with hepatocellular carcinoma patients, this assay was used in this thesis to monitor compounds modulating angiogenic activity.

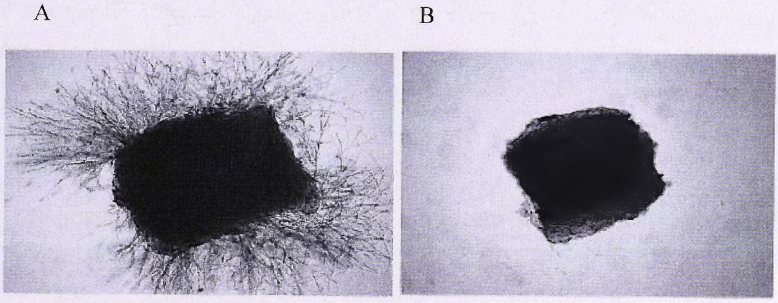


Figure 1.9. The rat aorta angiogenesis bioassay.

Untreated control (A), angiogenesis inhibited by PI-88 at 14 days post-treatment (B). From (Parish, Freeman et al. 1999).

1.11. Research objectives

The Djordjevic and Parish laboratories have previously identified a number of fractions from soybean (*Glycine max)* xylem sap that modulate angiogenesis using the *in vitro* rat aorta bioassay (Lauren Du Fall, ANU Honours thesis, characterisation of low molecular weight bioactive plant metabolites, unpublished data) (Du Fall 2009).

The aim of this PhD project was to screen for pro-angiogenic natural products from soybean xylem sap using an activity-guided bioassay to purify and isolate sufficient material to enable their identification. The active constituents of these fractions may have therapeutic value in situations where the formation of new blood vessels is described, such as in cardiovascular disease and wound healing.

To this end I planned to:

1) Fractionate and screen the soybean xylem sap for compounds that promote angiogenesis,

2) Purify and elucidate the structure of the bioactive compounds,

3) Determine their cellular mode of action using human cell lines, and

4) Investigate the pro-angiogenic activity and structure-activity relationship, at physiological concentrations, of a selection of flavonoids, including naringenin and genistein.

Chapter 2

Materials and Methods

2.1. Materials

The reagents used for chemical analyses were analytical grade mostly obtained from Sigma-Aldrich, St. Louis, MO and Ajax Chemicals, Auburn, NSW, Australia. Plant media were adjusted to the correct pH with 1M NaOH, 3M KOH and 1M HCL. Details of reagents, cells, media and buffers used throughout this thesis are listed in Table 2.1, 2.2 and 2.3.

2.2. Methods

2.2.1. Cultivation of soybean plants and treatments

The soybean (*Glycine max*) seeds (cv. Bragg) (CILR, Brisbane, Australia) were planted two per pot (30 cm × 10 cm × 10 cm) in vermiculite (Ausperl, Australia) under glasshouse conditions of 14 hours light (28 °C) and 10 hours dark photoperiod (25 °C). Plants were watered every day with ~ 200 mL of urban water for each pot and it was replaced with modified Herridge's nutrient solution (Herridge 1997) (1M KH_2PO_4 (pH to 6.0) 2 mM, K_2HPO_4 1mM, $MgSO_4.7H_2O$ 2 mM, KCl 1.5 mM, $CaCl_2.2H_2O$ 2.5 mM, ferric monosodium salt of EDTA (FeIII-EDTA) 1 mM, $H_3B\tilde{O}_3$ 2.86 g/L, $MnCl_2.4H_2O$ 1.812 g/L, $ZnCl_2$ 0.112 g/L, $CuCl_2.2H_2O$ 0.051 g/L, $Na_2MoO_4.2H_2O$ 0.024 g/L) from the second week of planting with application on every second day. The solutions were made up individually and mixed and diluted into 10 litres of water. Half of the plants were inoculated with the commercial soybean inoculum Nodulaid 100 (*Bradyrhizobium japonicum* strain CB 1809, Bio-Care Technology P/L Somersby NSW, 10^{10}/gram) at one week post-germination.

Uninoculated plants were treated with a range (1-10mM) of KNO_3 fertiliser and 10 mM KNO_3 which when added to other solutions mentioned above was determined to be sufficient to support growth until harvesting time at 4-6 weeks.

Table 2.1. General reagents used in this thesis.

General reagents	Supplier
Acetonitrile	Labscan Analytical Sciences, HPLC grade, 99.9%
ε-aminocaproic acid	Sigma-Aldrich, St. Louis, MO
Amphotericin B (fungizone)	Sigma-Aldrich, St. Louis, MO
Aprotinin	Boehringer, Mannheim, Germany
bFGF	R&D Systems, Minneapolis, MN
BSA	Sigma-Aldrich, St. Louis, MO
DMSO	Cambridge Isotope Laboratories, Andover, MA
Endothelial cell growth factor	Gibco BRL, Grand Island, NY
Endothelial cell growth supplement	Sigma-Aldrich, St. Louis, MO
Ethanol absolute	Chem Supply, Gilma, SA
Fibrinogen	Calbiochem, La Jolla, CA
Fibronectin (bovine plasma)	Invitrogen, Carlsbad, CA
Formic acid	Fluka, puriss. P , HPLC grade for mass spectrometry, ~ 98%
Gelatine (porcine skin)	Sigma-Aldrich, St. Louis, MO
Gentamycin	Sigma-Aldrich, St. Louis, MO
Glycerol	Ajax Chemicals, Auburn, NSW, Australia
Heparin (bovine lung)	Sigma-Aldrich, St. Louis, MO
HEPES	Sigma-Aldrich, St. Louis, MO
HIFCS	SAFC, Street Lenexa, KS
Hydrocortisone	Sigma-Aldrich, St. Louis, MO
L-glutamine	Sigma-Aldrich, St. Louis, MO
Matrigel	BD Biosciences, Bedford, MA
Milli-Q water (>18.2 MΩ.cm resistivity)	Millipore, Billerica, MA, USA
PD173074	Stemgent, San Diego, CA
Plasmin (human)	Sigma-Aldrich, St. Louis, MO
PSN (× 1000)	Penicillin G 30.072 g/L, streptomycin sulphate 50 g/L, neomycin sulphate 50 g/L
Rose Bengal pure A.R (dye)	Koch-Light Laboratories Ltd., Colnbrook, Berkshire, England
Thrombin	Sigma-Aldrich, St. Louis, MO
Thymidine (^{3}H)	MP Biomedicals, Solon, OH
Trypan Blue	BDH Poole Chemicals, London, England
Vitronectin (human)	Gibco BRL, Grand Island, NY

bFGF, basic-fibroblast growth factor; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HIFCS, heat inactivated foetal calf serum.

Table 2.2. Media and buffers used in this thesis.

Media and buffers	Composition
DMEM H16 medium	H16 powder 9.99 g/L (Gibco BRL, Grand Island, NY), $NaHCO_3$ 3.7 g/L
HBSS (× 10)	NaCl 100 g/L, KCl 5 g/L, $MgSO_4.7H_2O$ 1.25 g/L, $MgCl_2$ $6H_2O$ 1.25 g/L, $CaCl_2$ 1.4 g/L, glucose 12.5 g/L, Phenol Red 1 g/L, $Na_2HPO_4.2H_2O$ 3.9 g/L KH_2PO_4 3 g/L
M199 medium	M199 powder 9.5 g/L (Gibco BRL, Grand Island, NY), $NaHCO_3$ 2.2 g/L
MCDB 131	MCDB 131 Medium liquid (Gibco BRL, Grand Island, NY)
PBS	NaCL 8 g/L, $Na_2HPO_4.2H_2O$ 1.25 g/L, $Na_2HPO_4.H_2O$ 0.35 g/L
Trypsin/EDTA	4.25% trypsin ($Na_2HPO_4.2H_2O$ 0.075 g/L, KH_2PO_4 0.06 g/L), 5.3% versene (1% EDTA) in PBS

DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's balanced salt solution; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid.

Table 2.3. Cell lines, media and supplements used in this thesis.

Cell lines	Origin and details	Media and supplements
HUVEC	Human umbilical vein endothelial cells, primary cell line, adherent	M199, 20% HIFCS, 0.24 mg/mL gentamycin, 2 mM L-glutamine, 0.04-0.08 mg/mL ECGS, 0.135 mg/mL heparin
HMEC	Human micro vascular endothelial cell line, immortalised cell line, adherent	MCDB 131, 10% HIFCS, 2 mM L-glutamine, 0.1% PSN (0.03 g/L penicillin G, 0.05 g/L streptomycin sulphate, 0.05 g/L neomycin sulphate), 0.01 µg/mL endothelial cell growth factor, 1 µg/mL hydrocortisone
NIH-3T3	Mouse fibroblast cell line, adherent	DMEM, 10% HIFCS, 1mM L-glutamine, 2.5 mg/mL fungizone, 0.24 mg/mL gentamycin, 20 mM HEPES

HIFCS, heat inactivated foetal calf serum; ECGS, endothelial cell growth supplement; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2.2.2. Collection of soybean xylem sap

Soybean xylem sap was collected four-six weeks post-germination (Fig. 2.1A). Sap extraction was optimised by watering the plants one hour prior to extraction. The plants were decapitated with a scalpel blade at 5-8 cm above the vermiculite at the lowest section that can provide a secure fit for the plastic collection tubing (Fig. 2.1B). The collection procedure was as described by (Djordjevic, Oakes et al. 2007). The sap was collected over 0-7 and 7-24 hours. Negative pressure was provided by repositioning the 5 mL syringes during extraction hours and the plants left to bleed sap overnight. Sap exudation for each plant was estimated at 0.34 mL/hour over 24 hours. The extracted sap from 50 plants was pooled and lyophilized overnight (200-300 mT vacuum; Flexi-dry MP Microprocessor controlled bench top lyophiliser).

2.2.3. Compound purification from xylem sap

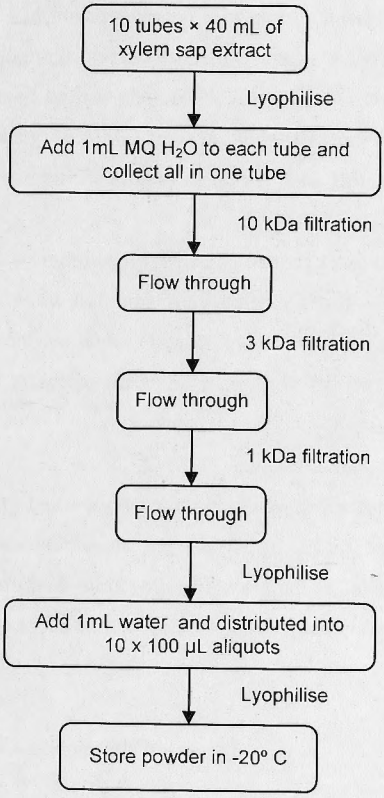
Molecular weight spin filters with different pore sizes (10 kilo Daltons (kDa), 3kDa and 1kDa) (Merck Millipore, Billerica, MA) were utilised to isolate the small molecules from the crude concentrated extract (Scheme 1). The 1 kDa filtrate was freeze dried, redissolved in 1 mL water (~ 400 fold concentration) and distributed into 10 × 100 µL aliquots for freeze drying and stored at -20 °C. Next, reverse phase high performance liquid chromatography (HPLC) with a C18 column was used to fractionate the filtered extract. The freeze dried samples were redissolved in 100 µL water and run on HPLC (2 × 50 µL injections representing 20 mL of xylem sap).

A

B

Figure 2.1. Extraction of xylem sap from soybean (*Glycine max*) plants.

Xylem sap was extracted from 4-6 week old soybean plants (A) by decapitating at ~ 5-8cm above the vermiculite and attaching a syringe by placing a piece of plastic tube over the stem. A 200µL pipetteman tip (→) was used to create the negative back pressure by holding the extended syringe in place (B).



Scheme 1. Preparation of xylem sap prior to HPLC purification.

2.2.4. High Performance Liquid Chromatography (HPLC)

Samples were subjected to chromatographic separation on a Shimadzu LC10-VP series HPLC using an Alltech Platinum C18 column (5µm, 250 × 4.6 mm) and guard cartridge run at 35 °C and eluted over 30 min with a linear gradient of 5% to 85% acetonitrile (Labscan Analytical Sciences, HPLC grade, 99.9%) (hold 5 min). Sample components were detected with a photodiode array (PDA) detector (190 – 800 nm). Water for HPLC analysis (>18.2 MΩ.cm) was supplied from a linked reverse osmosis and Milli-Q Gradient water purification system (Millpore, Billerica, MA, USA).

Peaks were collected by monitoring UV absorbance in real time at 254 nm at a flow rate of 1 mL/min. Fifty six fractions were collected from 2-30 min at 30 seconds intervals. Fractions were dried under nitrogen to remove acetonitrile, diluted to 200 µL with Milli-Q water and either screened for activity in the pro-angiogenic bioassay or stored at -70 °C.

The final purification of pro-angiogenic compounds was carried out on the Alltima C18 column as per the above method (injected 50 µl, 20 mL sap equivalent); however elution was over 15 min (5-20 min) with a linear gradient of 20% to 31% acetonitrile with final wash from 20 to 25 min with acetonitrile gradient of 31% to 85% (hold 5 min).

2.2.5. Rat aorta model angiogenesis bioassays

The *in vitro* rat aorta angiogenesis bioassay was used to screen for activity in the HPLC xylem sap fractions. Inoculated and nitrate treated plants were tested using a pro-angiogenic variant of an aorta test used to identify compounds that modulate angiogenesis (Parish, Freeman et al. 1999). The original method describes an anti-angiogenesis assay but to test for pro-angiogenic activity, the heat inactivated foetal calf serum (HIFCS) concentration in the culture medium is reduced from 20% to 5% (A Bezos, C Parish; unpublished method).

Thoracic aorta were excised from 5-9 month old female Fischer rats, rinsed in 2.5 µg/mL Hanks solution containing 1% amphotericin B (fungizone 250 µg/mL, Sigma) and cut into ~ 1mm wide sections. All the periadventitial fibroadipose tissue and blood

clots were removed. Dissecting and sectioning of the vessels were performed using a dissecting microscope. The assay was carried out in a 48 well culture plate (Costar, Corning, Lowell, MA) with 6 repeats for each sample. The vessel segment was embedded in a mix of 500 µL fibrinogen (3 mg/mL, bovine plasma, Calbiochem, La Jolla, California) in serum free medium M199 (GibcoBRL) and 2.5 µL aprotinin (5 µg/mL, Sigma) in PBS (phosphate buffer solution). The aprotinin prevents fibrinolysis of the vessel fragments. Then 15 µL of thrombin (50 NIH U/mL in 0.15 M NaCl: EC 3.4.21.5 bovine plasma: Sigma St Louis, MO) was added to each well and mixed rapidly to set the fibrin gel.

After clot formation, 500 µL of M199 medium (Life Technologies), with or without test compounds, supplemented with 20% or 5% HIFCS (SAFC, Street Lenexa, KS), 0.24 mg/mL gentamycin (Sigma-Aldrich, St. Louis, MO), 2.5 mg/mL fungizone (Sigma-Aldrich, St. Louis, MO), 1 mM L-glutamine (Sigma-Aldrich, St. Louis, MO) and 0.1% ε-aminocaproic acid (Sigma-Aldrich, St. Louis, MO) was added to each well. The plates were incubated at 37 °C in 5% CO_2 and the medium was changed on day 4 of culture. The vessel outgrowth was quantified manually as percent growth under a Nikon TMS inverted phase contrast microscope (Nikon Instrument Inc., Tokyo, Japan) at 40 × magnification on days 5-7 of culture. Two controls cultures were used: one received medium with the diluent and to the second the known anti-angiogenic agent, PI-88 (Parish, Freeman et al. 1999) was also added at 100 µg/ml. Percent growth is subject of estimation and was determined by the density and number of new vessels that had sprouted from the aorta ring in the field of view.

2.2.6. Liquid Chromatography Mass Spectrometry (LC-MS)

Mass spectrometry was performed using an Agilent 6530 Accurate Mass LC-MS Q-TOF (Santa Clara, CA, USA). Samples were subjected to ESI in the Jetstream interface in the positive and negative modes under the following conditions: gas temperature 300 °C, drying gas 4 L/min, nebulizer 35 psig, sheath gas temperature 350 °C and flow rate of 11 L/min, capillary voltage 3500 V, fragmentor 175 V, and nozzle voltage 1000 V. Samples (3 µL) were injected onto an Agilent Eclipse XDB-C18 column (2.1 × 50 mm; 1.8 µm) and xylem sap crude extract or HPLC purified pro-angiogenic compounds were eluted with a linear gradient from 10 to 50% mobile phase B in 8 min, then to 70% in 4 min (hold for 8 min) at a flow rate of 200 µL/min. Mobile phase A consisted of water

containing 0.1% formic acid while mobile phase B consisted of acetonitrile/water (9: 1 v/v) containing 0.1% formic acid. The instrument was run in extended dynamic range mode from *m/z* 100-3000 and data acquired by targeted collision induced dissociation (CID; N_2 collision gas supplied at 18 psi) MS/MS (2 spectra/s). Data were acquired and analysed with Agilent's MassHunter software.

2.2.7. Nuclear Magnetic Resonance Spectroscopy (NMR, performed by Tom Carruthers in the ANU Research School of Chemistry)

NMR spectroscopy experiments were recorded on either a Bruker AVANCE 800 or 600 spectrometer with TCI cryoprobe using D_2O as the solvent at 298 K. ^{1}H NMR chemical shifts in parts per million (ppm) are reported using the residual water solvent peak as an internal chemical shift reference (4.80 ppm).

^{1}H. 1D proton spectra were performed using solvent suppression via pre-saturation. 128 scans were taken of the 8.43 ppm spectral width.

^{1}H-DQF-COSY. A phase-sensitive 2D double quantum filtered COSY with pre-saturation was measured with 2K and 512 points were taken in the t_2 and t_1 proton dimensions respectively, sweep widths of 8.43 ppm each and 4 scans for each t_1 increment.

^{1}H-TOCSY. The phase sensitive 2D TOCSY with pre-saturation was measured with 4K and 212 points in the t_2 and t_1 proton dimensions respectively, a spectral width of 8.43 ppm in both dimensions, a MLEV mixing time of 60 ms and 4 scans for each t_1 increment.

^{1}H-^{1}H NOESY. A 2D homonuclear phase-sensitive NOESY with pre-saturation was run with 16384 and 512 points in the t_2 and t_1 proton dimensions, sweep widths of 8.43 and 11.11 respectively and 32 scans were measured for each t_1 increment.

^{13}C-HSQC. The phase-sensitive 2D ^{13}C-HSQC with adiabatic inversion pulses on ^{13}C was measured with 2K points in the proton dimension (t_2) and 1K points in the ^{13}C dimension (t_1), spectral widths of 8.43 ppm and 166 ppm respectively, and 8 scans for each t_1 increment. ***^{13}C-HMBC.*** The phase-sensitive 2D ^{13}C-HMBC included a two-fold

low-pass *J*-filter to suppress one-bond correlations. 4K points in the proton dimension (t_2) and 857 points in the ^{13}C dimension (t_1) were measured with spectral widths of 8.43 ppm and 222 ppm respectively with 32 scans for each t_1 increment.

2.2.8. Cell Culturing

2.2.8.1. Subculturing mammalian cell lines

Three mammalian cell lines were used in this thesis. The human umbilical vein endothelial cells (HUVEC), a human microvascular endothelial cell line (HMEC) and a NIH-3T3 mouse fibroblast line were each cultured in specific media described in Table 2.3 and incubated in a Hepa-filtered IR incubator (Forma Scientific, Marietta, OH) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were not re-assessed after thawing; however, cells were routinely tested for contamination by Mycoplasma.

The cells were routinely passaged every two to three days according to their cell proliferation rate and subcultured when they achieved 80% confluence. The number of passaging for HUVECs was not more than 8 and it was less than 16 for HMEC and 3T3 mouse fibroblasts. The HMEC and 3T3 mouse fibroblasts were cultured in 75 and 175 cm^2 flasks (Nunc, Roskilde, Denmark) and HUVEC cultured in 0.1% gelatine-coated 75 cm^2 flasks (Corning, Lowell, MA). Since all these cell lines are adherent, cell monolayers were released for sub-culturing with trypsin/EDTA mixture (PBS/0.05% EDTA/0.00025% Trypsin). Culture supernatants were removed from culture flasks and the adherent cells washed once with PBS to remove dead cells and residual medium. Into a 75 mL flask, 7.5 mL of pre-warmed trypsin/EDTA mixture at 37 °C was added and incubated for 3-5 min at 37 °C in 5% CO_2. Then, the flask was gently tapped to lift adherent cells. Once adherent cells were dislodged, 7.5 mL of culture medium was added to stop the action of the trypsin (proteolytic cleavage of cell surface receptors). The cells were then transferred to a 50 mL centrifuge tube and an extra 5 mL of culture medium was added to the flask as a final wash, which was also transferred to the centrifuge tube. The cells were centrifuged at 290 g for 5 min, the supernatant discarded and the cell pellet re-suspended in a recorded volume of culture medium. Finally, cells were sub-cultured at an appropriate cell density. For 175 mL flasks, all the media volumes were doubled.

2.2.8.2. Cell counting and viability

A 10 µL cell suspension of known volume was diluted 1: 1 with 0.1% Trypan Blue (BDH Poole Chemicals, London, England) and loaded into a haemocytometer chamber and viewed at 20 × magnification under a standard Olympus B light microscope (Olympus Optical, Tokyo, Japan) to assess the cell number and viability.

2.2.9. Cryopreservation of mammalian cell lines

The cells were collected as described in section 2.2.8.1 and re-suspended in culture medium containing 6% dimethyl sulphoxide (DMSO) (Cambridge Isotope Laboratories, Andover, MA) and stored in liquid nitrogen until use.

2.2.10. Cell proliferation assay (mitogenic assay)

The HUVEC and 3T3 fibroblast proliferation assays were based on a determination of ^{3}H-thymidine incorporation. The 3T3 fibroblast cells were cultured into 96-well culture plates (Linbro, Aurora, OH) at 3 × 10^{3} cells/well in 100 µL/well DMEM, supplemented with 10% HIFCS, 1 mM L-glutamine, 2.5 mg/mL fungizone, 0.24 mg/mL gentamycin, 20 mM HEPES (Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C in 5% CO_2 for 2-5 days until confluent. The confluent monolayers were then serum starved for 48 hours by changing the medium to serum free DMEM (100 µL/well). Subsequently, 100 µL/well of test compound in serum free DMEM medium with or without bFGF (R&D Systems, Minneapolis, MN, USA) was added to each well. Then 0.5 µCi/well of ^{3}H-thymidine (MP Biomedicals, Solon, OH) was added and the cells incubated for the final 24 hours. Incubation was stopped by freezing and thawing the cultures three times. Finally, thawed cultures were harvested using a 96-well cell Filtermate 196 harvester (Packard Bioscience, Meriden, CT), with EasyTab™-C self-Aligning Filters (Packard Bioscience, Meriden, CT) and the radioactivity incorporated by the cells counted with a Topcount®NXT™ Microplate Scintillation & Luminescence Counter (Packard Bioscience, Meriden, CT).

HUVEC were cultured in M199 medium supplemented with 20% HIFCS, 0.24 mg/mL gentamycin, 2 mM L-glutamine, 0.04 mg/mL endothelial cell growth supplement (ECGS) (Sigma-Aldrich, St. Louis, MO) and 0.135 mg/mL heparin (Sigma-

Aldrich, St. Louis, MO) in gelatine-coated 96-well plates and incubated for 4 days at 37 °C in 5% CO_2 to reach confluence. The cells were then serum starved for 24 hours. Serum starved cells were incubated in serum free medium with or without the test compounds for another 24 hours before adding ^{3}H-thymidine. Incubation was stopped with adding 100 µL/well of trypsin/EDTA to lift the cells from the gelatine layer with 100 µL/well of M199 medium supplemented with 20% HIFCS to neutralise trypsin enzymatic activity. Finally, plates were frozen and thawed 3 times and cells were harvested as described above and ^{3}H-thymidine incorporation determined.

2.2.11. Cell migration assay (wound healing assay)

The IncuCyte™ live-cell imaging system (Essen BioSciencen, Ann Arbor, MI) was employed for this assay which provides a 96-well wound making tool, automated image acquisition software (IncuCyte) and image processing metrics (IncuCyte) to provide a reproducible, label-free, kinetic assay for cell migration.

HMEC cultures were added to 96-well ImageLock Essen plates (Essen BioSciences, Ann Arbor, MI) at 2.5 × 10^4 cells/well in 100 µL/well of MCDB 131 medium supplemented with 10% HIFCS, 0.1% PSN (0.03 g/L penicillin G, 0.05 g/L streptomycin sulphate, 0.05 g/L neomycin sulphate), 2 mM L-glutamine, 0.01 µg/mL endothelial cell growth factor (ECGF), (Gibco BRL, Grand Island, NY) and 1 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO) and incubated for 2 days at 37 °C in 5% CO_2 to reach confluence.

After removing the media from each well, a wound was then made in the HMEC monolayer using the 96-pin WoundMaker device to produce precise and reproducible wounds by gently removing the cells. The use of Essen ImageLock plates ensured that the wounds were automatically located and registered by the IncuCyte software for imaging and data recording.

Each well was then rinsed twice with 100 µL/well of culture medium to prevent dislodged cells from settling and re-attaching. Then, 100 µL/well of medium, with or without test compound, was added to each well and the plate was placed inside the IncuCyte. Wound images were then captured and saved at 2 hours intervals until control wounds recovered completely. The data were analysed either by the IncuCyte

software package or extracted by three integrated metrics (wound with, wound confluence, relative wound density) and analysed by other statistical softwares.

2.2.12. Endothelial cell tube formation assay

HMEC and HUVEC cultures were used in *in vitro* endothelial tube formation assays. Matrigel (BD Biosciences, Bedford, MA) was thawed overnight at 4 °C and then plated into ice-cold 96-well ImageLock Essen plates at 50 µL/well using pre-cooled pipettes, tips and tubes. The plates were incubated at 37 °C in 5% CO_2 for 1 hour to allow the Matrigel to form a stable gel. HUVEC at 4×10^4 cells/well in 100 µL of M199 medium supplemented with 20% HIFCS, 0.24 mg/mL gentamycin, 2 mM L-glutamine, 0.04 mg/mL ECGS, and 0.135 mg/mL heparin with or without test compounds were added and Matrigel cultures placed inside the IncuCyte and incubated at 37 °C for 24 hours.

A hundred microlitre of HMEC at 5×10^4 cells/well in MCDB 131 medium supplemented with 10% HIFCS, 0.1% PSN, 2 mM L-glutamine, 0.01 µg/mL ECGF, and 1 µg/mL hydrocortisone ± test compounds was added onto Matrigel. Tube formation images were obtained at 2 hours intervals using the IncuCyte™ live-cell imaging system with phase-contrast ImageLock scan type. The images were analysed by measuring percentage of denuded area and number of sprouting cells at early stages of tube formation (4 hours) and number of tubes and total length of tubes formed at later stages of tube formation (6 hours) in each well using IncuCyte and NIH ImageJ softwares.

2.2.13. Rose Bengal cell adhesion assay

The cell adhesion assay performed in this thesis is based on a method developed by (O' Neill and Parish 1983) to measure the interaction of antibodies with cell surface antigens using an automated colorimetric assay.

A 96 round bottom (U-well) culture plate (Costar, Corning, Lowell, MA) was first coated either with bovine plasma fibronectin (Invitrogen, Carlsbad, CA) or purified human vitronectin (Gibco BRL, Grand Island, NY) at 50 µL/well at selected concentrations ranging from 0.313 to 10 µg/mL and incubated overnight at 4 °C. The next day, the solution in the wells was flicked off and the wells then washed by

submerging the plate twice in a PBS bath (flicking off PBS in between washes). The non-specific binding sites were then blocked by incubation with 200 µL/well of 1% BSA in Hank's balanced solution (HBSS) for 1 hour at 37 °C before flicking off the supernatant. HMEC (5×10^4 cells/well) were added in 100 µL/well of serum free culture medium supplemented with 0.1% BSA with or without test compounds. The plates were then incubated either for a time course study (2.5-30 min) or for 60 min at 37 °C. The plates were then flicked to remove unbound cells and 100 µL/well of 0.25% (w/v) Rose Bengal dye (Koch-Light Laboratories Ltd., Colnbrook, Berkshire, England) in PBS was added and allowed to stain the bound cells, for 3 min at room temperature. The un-absorbed dye was then removed by flicking the plate and the plate washed twice by submerging it in separate PBS baths, with the PBS being changed between washes.

The plate was then allowed to drain and 200 µL/well of 50% ethanol in PBS added and mixed to liberate dye from the cells. Non-specific binding of the dye to fibronectin/vitronectin-coated and uncoated wells in the presence and absence of cells was also determined and optical density values subtracted from test values. The relative number of bound-cells in each well was quantified by determining each well's optical density (OD) at λ_1=540 nm and λ_2=650 nm, on a Thermomax microplate reader. The data were then analysed by GraphPad prism 5.04 software.

2.2.14. Statistical significance

Data and results are reported as means ± SEM (standard error of the mean) unless noted otherwise. Statistical significance was measured using a two-tailed unpaired *t*-test and Student–Newman–Keuls test after one-way ANOVA comparing the sample treated group and the untreated control group using the GraphPad prism 5.04 software (GraphPad Software, San Diego, CA). Also, two-way ANOVA data analysis followed by Bonferroni post-test was applied to some graphs to compare each column to all the other columns. *P* values less than 0.05 were considered statistically significant.

Chapter 3

Purification and Structural Elucidation of Two Low Molecular Weight Pro-angiogenic Molecules from *Glycine max*

3.1. Introduction

Natural products derived from plants have intrinsic broad scientific interest but most particularly have been used as therapeutics. For example, the bark of the willow tree (*Salix sp.*) contains salicylic acid. The willow tree bark was used by the ancient Greeks and native Americans to treat a wide range of ailments (Rishton 2008). The salicylic acid present in the willow tree bark is not only a plant defence hormone but also the precursor of aspirin, a common over-the-counter analgesic that can also be used to treat inflammation and to inhibit platelet aggregation (Mahdi, Mahdi et al. 2006). More recently, the Pacific yew tree (*Taxus brevifolia*) (Wani, Taylor et al. 1971) and the French lilac (*Galega officianalis*) were the source of the active compounds in the drugs taxol and the biguanide class of antidiabetic, respectively, used in the treatment of breast cancer (Witters, Santala et al. 2003). Other plant derived anticancer drugs include; vincristine, vinblastine and camptothecin (Pezzuto 1997; Gordaliza 2007). Many natural products have pharmacological effects in humans and therefore may have potential therapeutic applications and commercial value.

Pharmacologically active compounds in plants often exist at very low concentrations and may include different classes of chemicals with a range of different polarities and solubilities (Tu, Jeffries et al. 2010). The challenge that this presents in developing an extraction and purification protocol is complicated since plant tissues represent a very complex matrix from which these materials must be retrieved.

One strategy that enhances the potential for success in identifying biologically active components in fractionated plant material is to couple a purification strategy with an activity guided assay (Li and Vederas 2009). If the assay is sensitive and robust enough to detect biologically active components in crude plant fractions, it can also be used subsequently to follow the active components through further purification steps (Butler 2004). These purification steps often involve various forms of chromatography that separate molecules according to size, charge or hydrophobicity such as size exclusion chromatography (SEC), molecular weight spin filters, ion exchange or reverse phase chromatography (Cannell 1998; Wu, Yang et al. 2008). Once the active fractions have been fully purified in sufficient quantities then the classical techniques of nuclear magnetic resonance (NMR) and mass spectrometry (MS) can be applied to the problem of elucidating the structure of the active components (Kingston 2011).

The laboratory I worked in has a background in identifying biologically active plant components in xylem sap that are involved in long distance signalling (Djordjevic, Oakes et al. 2007). Since quantities of xylem sap were available for screening this material was utilised in my research to screen for biologically active components that have activity with mammalian cells. The choice of xylem sap also had other advantages since it is known to be a rich source of metabolites (Krishnan, Natarajan et al. 2011), can be isolated relatively easily and it is free from pigments and cell wall material that are major components of plant tissue extracts. In addition, preliminary work identifying biologically active components in this material had been undertaken by Lauren Du Fall in 2009 (Du Fall 2009). In this work, the xylem sap was initially fractionated by size exclusion chromatography and a bioactive fraction was found in the low molecular weight eluate (<1 kDa). This low molecular weight fraction was then subject to reverse phase HPLC chromatography on a C18 column and further testing showed this fraction to contain compounds that could promote or inhibit angiogenesis.

A well understood, frequently utilised *in vitro* bioassay (Auerbach, Lewis et al. 2003) was used to monitor the activity of the various fractions derived from soybean. This assay has been used by researchers in the Parish laboratory to screen for anti-angiogenic compounds, with the new anti-angiogenic, sulphated oligosaccharide, drug PI-88 (US6271215, also called Muparfostat) being identified (Parish, Freeman et al. 1999). PI-88 is currently undergoing a phase III clinical trial in hepatocellular carcinoma patients (Liu, Lee et al. 2009). However, since a large number of anti-angiogenic compounds were already in different stages of commercial development (Li, Li et al. 2012), this angiogenesis assay was then modified to enable determination of pro-angiogenic activity of Nod factors (Djordjevic, Bezos et al. 2013) as well as screening of plant natural products by DuFall (Du Fall 2009) and in this thesis.

3.2. Research aims

This chapter describes the further investigation of the xylem sap of *Glycine max* using activity-guided fractionation based on the *in vitro* rat aorta angiogenesis bioassay. One of the problems with the initial investigation was the failure to isolate enough material for examination by nuclear magnetic resonance (NMR). In order to increase the throughput of the purification process it was decided to replace the size exclusion chromatography with molecular weight cut-off spin columns as these were potentially capable of readily processing larger volumes of the xylem sap, in parallel, and at a faster rate. Studies were also initiated to maximise the yield of the active compounds in the xylem sap and different planting regimes and conditions for growth for the glasshouse grown soybeans were examined. It was expected that a combination of both enrichment and higher throughput would allow sufficient material to be accumulated to successfully complete the structural elucidation, using liquid chromatography mass spectrometry (LC/MS) and nuclear magnetic resonance spectrometry (NMR), of the pro-angiogenic molecules.

3.3. Research objectives

1) Identify and isolate the pro-angiogenic compounds less than 1 kDa from xylem sap using *in vitro* pro-angiogenesis rat aorta bioassay.

2) Purify the compounds with pro-angiogenic activity.

3) Determine the structure of pro-angiogenic molecules using LC/MS and NMR.

4) Confirm the determined structure of natural compounds with synthetic versions.

3.4. Results

3.4.1. Soybean cultivation optimisation

As soybeans (*Glycine max*) are subtropical plants, they were cultivated in a glasshouse between spring and autumn using artificial lighting to extend day length was necessary. However, planting in mid- to late-summer seemed to result in the most robust growth and this was reflected in the enhanced yield of the bioactive compounds (Table 3.1) (Fig 3.1, 3.2, 3.3A and 3.3B).

Plants were either inoculated with *Bradyrhizobium* or uninoculated and supplemented with nitrate fertiliser (1mM or 10 mM KNO_3). In order to establish the optimal amount of nitrate required for growth, uninoculated soybean plants were treated with varying concentrations of nitrate (1 or 10 mM KNO_3). It was determined that 200 mL of 10 mM of KNO_3 solution every two days was sufficient to support growth for 4-6 weeks. Plants were decapitated at 6 weeks and the xylem sap extracted over 0-7 and 7-24 hours.

3.4.2. Purificatiou of small molecules of xylem sap

In the initial experiments, size exclusion chromatography (SEC) using the Superdex™ Peptide HR 10/30 size exclusion column was used to fractionate the xylem sap material (Oakes, Bezos, Parish & Djordjevic, unpublished data; DuFall 2009) to purify thirty one fractions (1mL) collected from reverse phase HPLC (C18). Two pro-angiogenic and three anti-angiogenic fractions were identified. The late elution of these active fractions suggested that firstly, the active compounds were <1000 Da and secondly, a mixed mode of separation was occurring (i.e. size exclusion and adsorption to the dextran/cross-linked agarose matrix; see for example (Schluter and Zidek 1993). Later, LC/MS analysis of one of the pro-angiogenic molecules, showed the molecular mass of 375 Da. Therefore, the aim of this research described in this thesis was focused on purifying molecules less than 1 kDa on a large enough scale to complete the structural analysis of the isolated angiogenic compounds.

Table 3.1. Volume of extracted xylem sap from soybeans planted at different times and grown under different conditions.

Number of soybean plants	Season of planting	Season of harvesting	*Rhizobium* inoculated or KNO_3 supplemented	Volume of extracted xylem sap
100	Mid-October	End-November	*Rhizobium* inoculated	~ 700 mL
100	Mid-October	End-November	1 mM KNO_3	~ 600 mL
100	Mid-October	End-November	10 mM KNO_3	~ 700 mL
100	Mid-January	End-February	10 mM KNO_3	~ 750 mL
100	Mid-March	End-May	10 mM KNO_3	~ 400 mL

In order to maximise the amount of material, new purification methods were developed to isolate small molecules from the soybean crude extract. A succession of 10, 3 and 1 kDa of molecular weight spin filters was used to isolate the low molecular weight fractions. However, it was found that the 1 kDa filter retained a proportion of biologically active molecules, so the isolation steps were limited to using only the 10 and 3 kDa filters (Fig. 3.3B and 3.3C). This had the advantages of decreasing the spinning time at a reduced cost purification method. Thus, the 3 kDa spin filtration following a 10 kDa spin filtration appeared to offer the best purification of the bioactive compounds. The small molecule fraction was lyophilised by freeze-drying for storage at -80 °C.

3.4.3. Reverse phase HPLC purification of xylem sap small molecules

Different combinations of water, polar and non-polar organic solvents (Diethyl Ether, Ethyl acetate and Acetonitrile) were tested to determine the optimal liquid composition for resuspending the lyophilised filtrate for chromatographic separation by HPLC. The use of all except water showed incomplete re-suspension of the dried 3 kDa xylem sap filtrate. Therefore, deionised water (>18.2 MΩ.cm) was used to resuspend the lyophilised material prior to HPLC fractionation.

The concentrated reconstituted xylem sap was fractionated by C18 reverse phase chromatography into 30s fractions over a 5%-85% acetonitrile gradient and all the fractions were screened for pro-angiogenic activity (Fig. 3.1, 3.2 and 3.3). Three fractions (***FK1***, ***FK2*** and ***P6***) from the 10mM KNO_3 treated soybean extracts were selected for further analysis. The UV absorbance of both ***FK1*** and ***FK2*** were essentially identical with UV absorbance maxima (λ_{max}) at 265 nm and a shoulder at 298 nm, suggesting some structural similarity. The UV absorbance of ***P6*** had a λ_{max} at 283 and 343 nm, as previously described by (Du Fall 2009) (Fig. 3.4). These UV absorbances are characteristic of phenols and conjugated double bond systems.

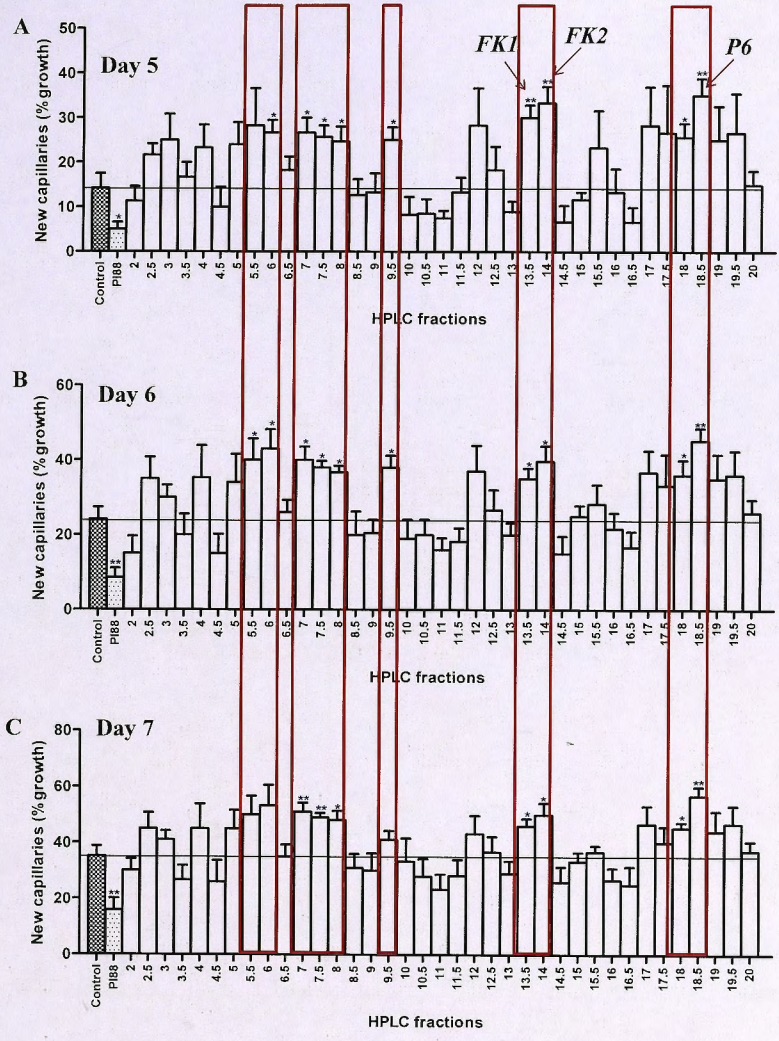


Figure 3.1. Pro-angiogenesis rat aorta assay on *Bradyrhizobium* inoculated xylem sap HPLC fractions.

HPLC eluate (2-20 minutes) was collected at 30 seconds intervals, then dried down to 100 µL and analysed. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5 (A), 6 (B) and 7 (C). PI-88 has clinically proven anti-angiogenic activity and was used as a negative control. Data analysis was performed by unpaired t-test, comparing each group to the control. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01, *** ≤ 0.001. Pro-angiogenic active fractions (□).

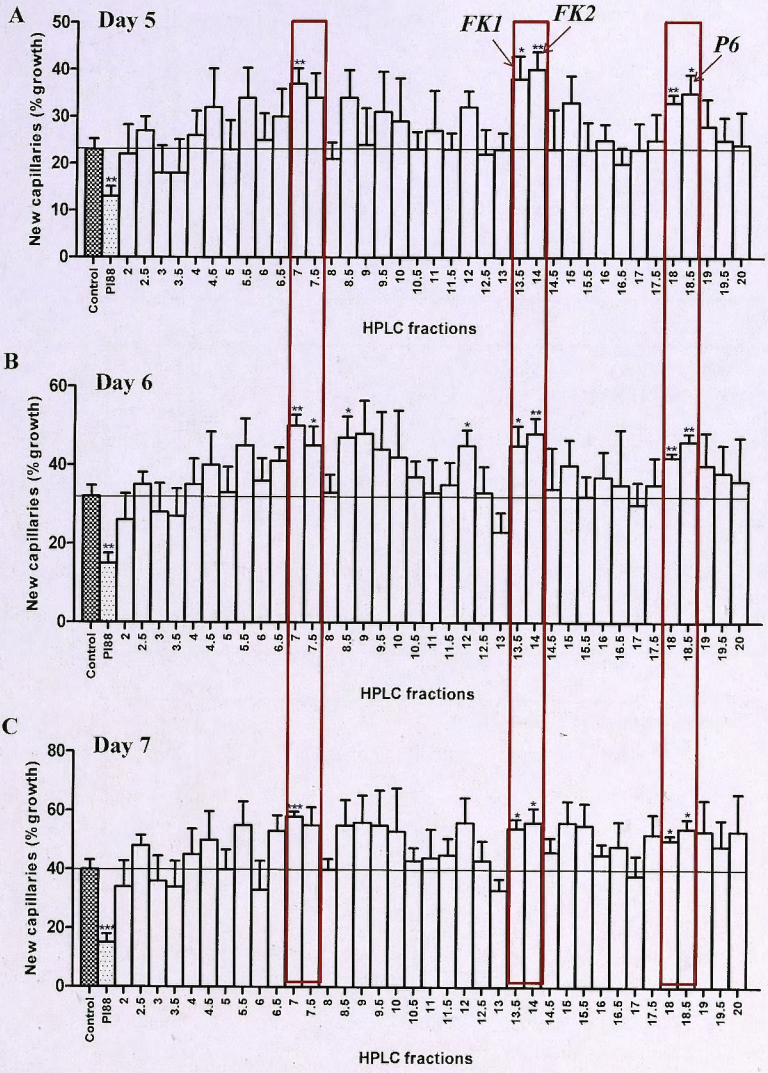


Figure 3.2. Pro-angiogenesis rat aorta assay on 10 mM KNO_3 xylem sap HPLC fractions.

HPLC eluate (2-20 minutes) was collected at 30 seconds intervals, then dried down to 100 µL and analysed. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5 (A), 6 (B) and 7 (C). PI-88 has clinically proven anti-angiogenic activity and was used as a negative control. Data analysis was performed by unpaired t-test, comparing each group to the control. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01, *** ≤ 0.001. Pro-angiogenic active fractions (□).

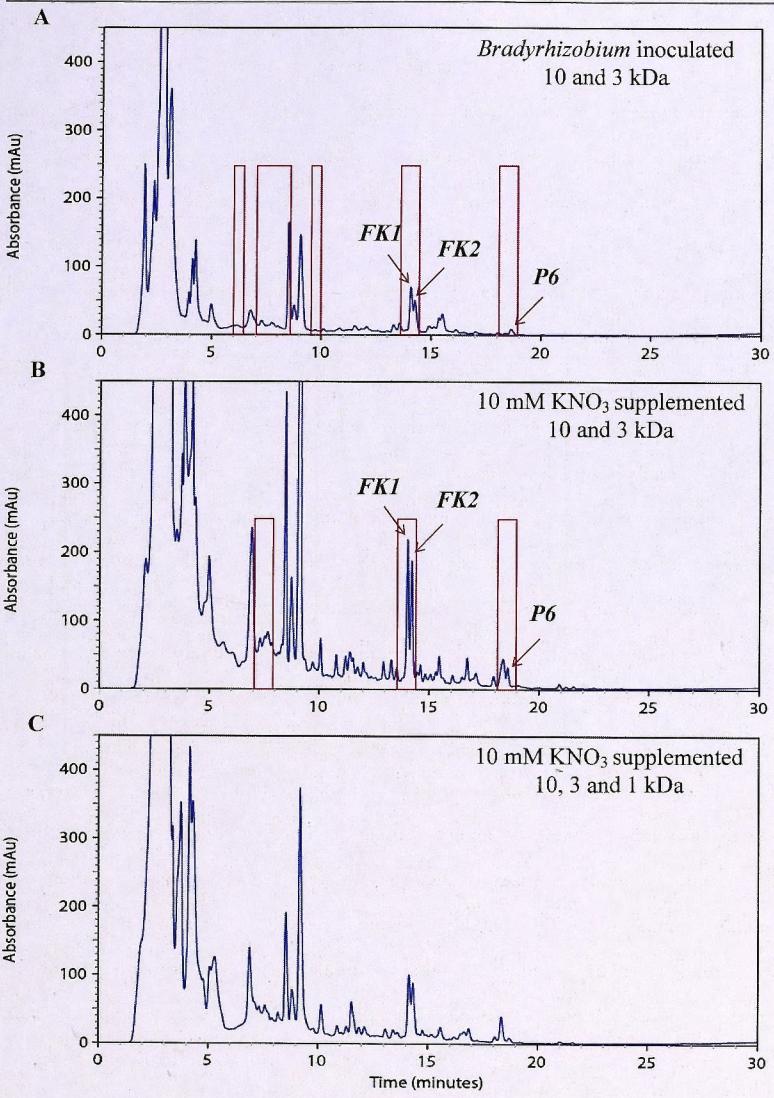


Figure 3.3. HPLC chromatograms of xylem sap from *Bradyrhizobium* inoculated and nitrate supplemented soybean plants.

HPLC separation of 3 kDa filtrate xylem sap from the *Rhizobium* inoculated (A) and un-inoculated, 10 mM nitrate supplemented (B) soybeans. HPLC separation of 1 kDa filtrate xylem sap from 10 mM nitrate supplemented soybeans (C). An equivalent amount of material (50 µl injection equivalent to 20 mL sap) was loaded in all injections. Absorbance was monitored at 254 nm. Fractions were collected at 30 s intervals from A and B and tested by the rat aorta pro-angiogenesis bioassay. Pro-angiogenic active fractions (□).

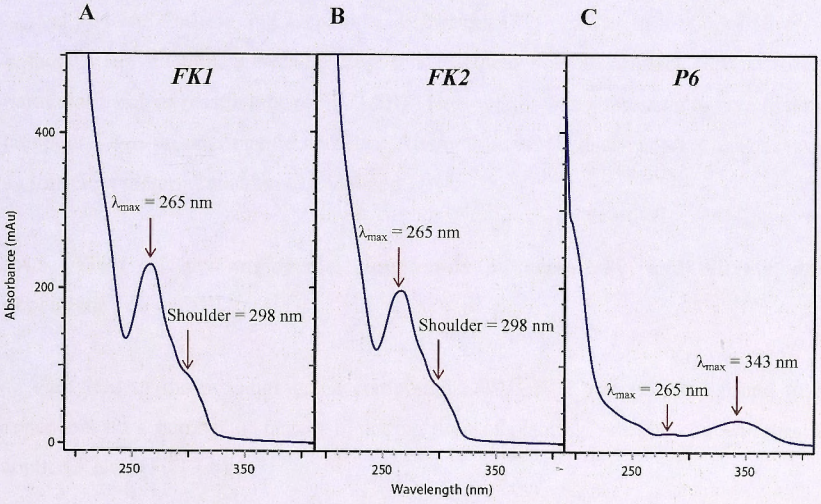


Figure 3.4. UV absorbance of pro-angiogenic HPLC fractions *FK1*, *FK2* and *P6*.

FK1 (A) and ***FK2*** (B) UV absorbance spectra were essentially identical with λ_{max}= 265 nm and shoulder at 298 nm. The UV spectra of ***P6*** was characterised by a λ_{max} at 283 and 343 nm. Absorbance was monitored at 254 nm.

3.4.4. Hydrolysis experiments

To investigate the possibility that ***FK1***, ***FK2*** or ***P6*** may be glycosylated, the purified material was subject to acid hydrolysis with 1M HCl in a plastic tube immersed in boiling water for an hour. HPLC analysis of the reaction mixtures showed the disappearance of each of ***FK1***, ***FK2*** or ***P6*** from the HPLC trace and their replacement by a single, later eluting peak (Fig. 3.5A) with a UV absorption spectra characterised by λ_{max} at 204 and 260 nm and a shoulder at 298 nm (Fig. 3.5C). In a blank hydrolysis without a sap fraction, it became clear that this peak was an artefact derived from a particular batch of plastic tubes (Fig. 3.5B). However, it was interesting to note that this compound had pro-angiogenic activity. Attempts to NMR analysis were unsuccessful as sufficient material could not be isolated.

3.4.5. Yield of pro-angiogenic compounds is seasonally and nitrate dose dependent

The yield of the pro-angiogenic compounds ***FK1***, ***FK2*** and ***P6*** were found to be dependent on a number of factors including time of planting, and the concentration of applied KNO_3 (Fig. 3.6).

The optimal yield of ***FK1***, ***FK2*** and ***P6*** was found to be from plants grown in January/February and supplemented with 10 mM KNO_3 (Fig. 3.6C). However, for some reasons, not fully understood, the yield of ***P6*** was subject to a large degree of variation. For this reason, it was decided to concentrate on only isolating ***FK1*** and ***FK2*** in sufficient amounts for NMR and MS analysis.

3.4.6. Final purification of bioactive fractions for structural analysis

The HPLC separation of ***FK1*** and ***FK2*** was optimised to enable the isolation of pure ***FK1*** and ***FK2*** prior to NMR and MS analysis (Fig. 3.7). A total of 458 µg of ***FK1*** and 387 µg of ***FK2*** were purified. This represented 2 L of sap from 300 6 week old soybeans. The pro-angiogenic activity of the purified ***FK1*** and ***FK2*** were then confirmed by bioassay before NMR and MS analysis (Fig. 3.8).

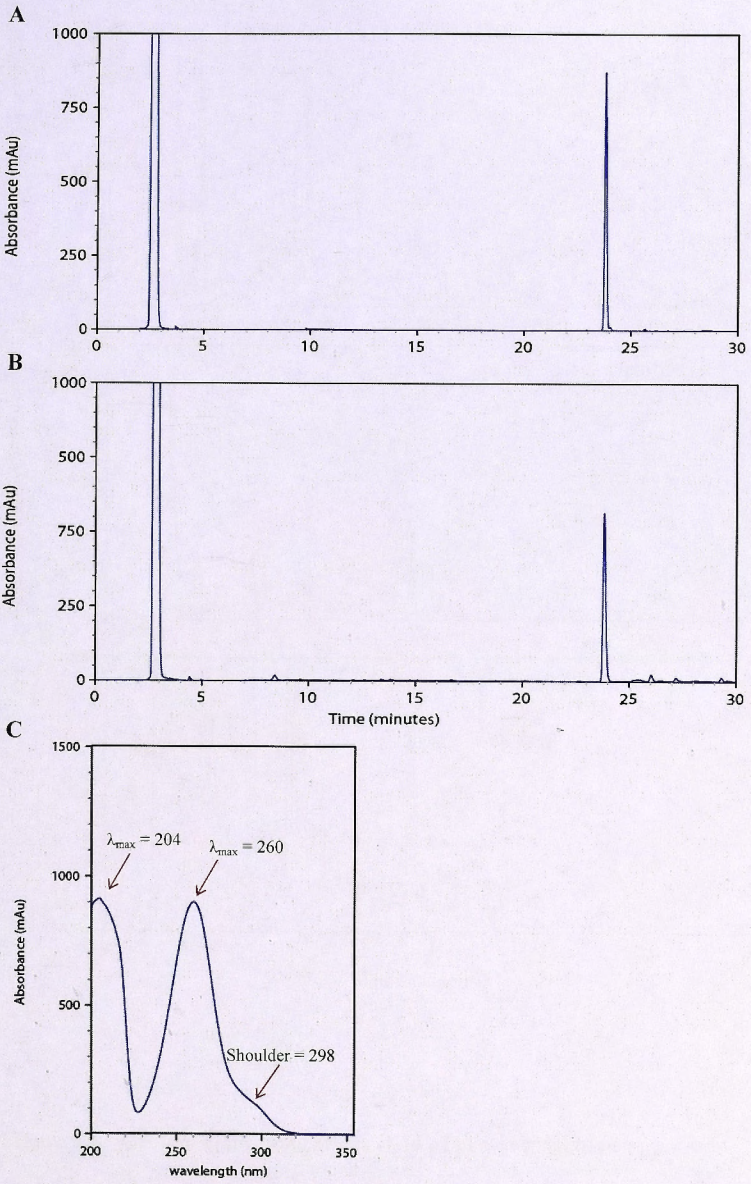


Figure 3.5. HPLC chromatogram and UV absorbance spectra of the 1M HCl hydrolysis product from the plastic tubes.

HPLC separation of hydrolysed a plastic tube (A) and hydrolysed ***FK1*** (B) and UV absorbance spectra (C) of the artefact derived from the 1M HCl hydrolysis. This proved to derive from the plastic container in which the hydrolysis was performed.

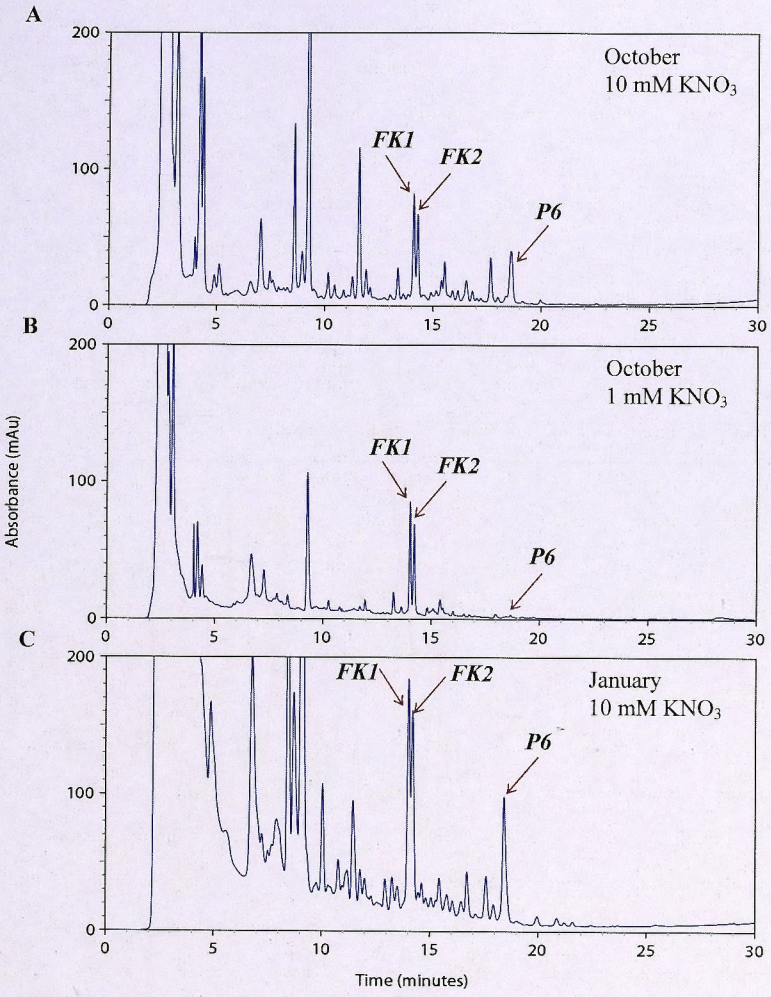


Figure 3.6. Growth conditions affect yield of pro-angiogenic compounds.

HPLC chromatograms of xylem sap extracted from 6 weeks old soybeans grown under different conditions (A), seeds were planted in October and supplemented with 10 mM nitrate (B), seeds were planted in October and supplemented with 1 mM nitrate (C), seeds were planted in January and supplemented with 10 mM nitrate. An equivalent amount of material (50 µL injection equivalent to 20 mL sap) was loaded in all injections. Absorbance was monitored at 254 nm.

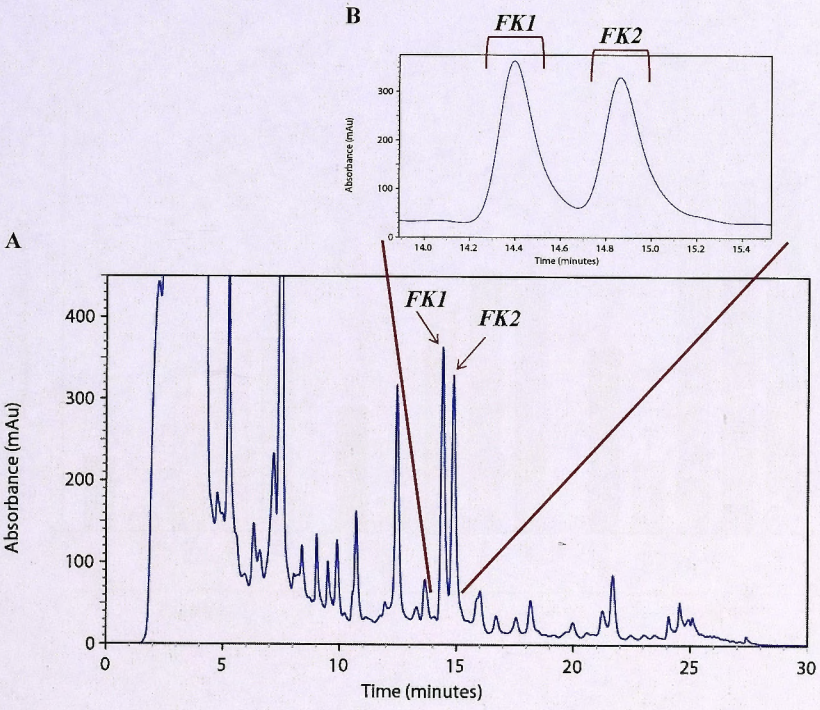


Figure 3.7. Semi-preparative purification of *FK1* and *FK2* by HPLC.

High resolution separation of ***FK1*** and ***FK2*** from xylem sap was optimised by modifying the analytical HPLC method (A) and ***FK1*** and ***FK2*** peaks were individually collected (B). An equivalent amount of material (50 µL injection equivalent to 20 mL sap) was loaded. Absorbance was monitored at 254 nm.

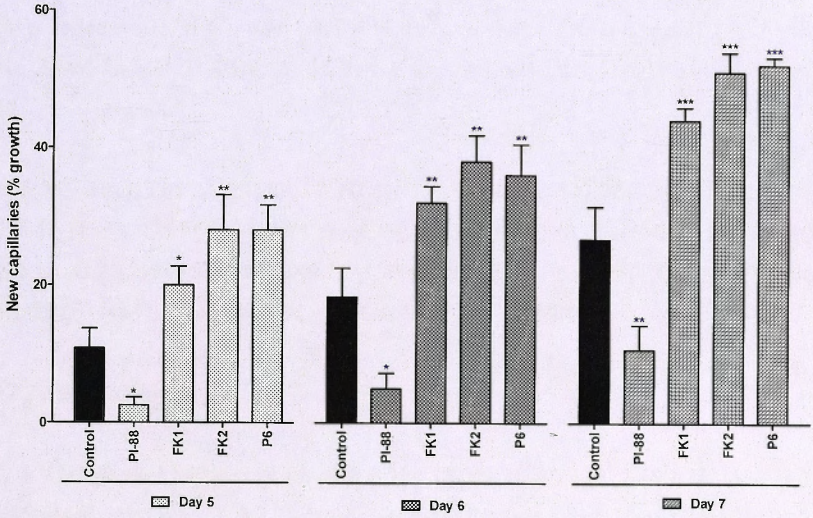


Figure 3.8. Pro-angiogenesis rat aorta assay on purified *FK1, FK2* and *P6* from xylem sap.

FK1, ***FK2*** and ***P6*** fractions from xylem sap were collected from HPLC, then dried down to 100 μL and analysed. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5, 6 and 7. PI-88 has clinically proven anti-angiogenic activity and was used as a negative control. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01, *** ≤ 0.001.

3.4.7. NMR analysis of purified *FK1* and *FK2*

FK1 chemical shifts

δ(^{1}H, D_2O, 800 MHz) 6.88 (d, J_4=2.0 Hz, 1H, **H-2**), 6.74 (d, J_3=8.1 Hz, 1H, **H-5**), 6.86 (dd, J_3= 8.1 Hz, J_3=1.9 Hz, 1H, **H-6**), 4.7 (overlapped m, 2H, **H-7**), 4.66 (m, 1H, **H-8**), 4.07 (dd, J_2= 12.3 Hz, J_3= 2.8 Hz, 1H, **H-9a**), 3.94 (dd, J_2= 12.3 Hz, J_3= 6.7 Hz, 1H, **H-9b**), 3.68 (s, 3H, **H_3-10**), 6.98 (bs, 1H, **H-2′**), 6.97 (overlapped s, 1H, **H-5′**), 6.97 (overlapped s, 1H, **H-6′**), 6.56 (dt, J_3=16.0 Hz, J_4=1 Hz, 1H, **H-7′**), 6.31 (dt, J_3=15.9 Hz, J_3=6.0 Hz, 1H, **H-8′**), 4.25 (dd, J_3=6.0 Hz, J_4=1.0 Hz, 2H, **H_2-9′**), 3.70 (s, 3H, **H_3-10′**).

δ(^{13}C, D_2O, 201 MHz) 130.60 (**C-1**), 111.38 (**C-2**), 147.72 (**C-3**), 147.2 (**C-4**), 115.42 (**C-5**), 121.13 (**C-6**), 72.54 (**C-7**), 83.56 (**C-8**), 61.52 (**C-9**), 55.69 (**C-10**), 131.13 (**C-1′**), 109.78 (**C-2′**), 149.22 (**C-3′**), 146.94 (**C-4′**), 116.56 (**C-5′**), 119.45 (**C-6′**), 130.61 (**C-7′**), 126.76 (**C-8′**), 62.29 (**C-9′**), 55.46 (**C-10′**).

FK2 chemical shifts

δ(^{1}H, D_2O, 800 MHz) 6.94 (d, J_4=1.9 Hz, 1H, **H-2**), 6.77 (d, J_3=8.1 Hz, 1H, **H-5**), 6.85 (overlapped dd, J_3= 8.1 Hz, J_3=1.7 Hz, 1H, **H-6**), 4.89 (d, J_3=5.5 Hz, 2H, **H-7**), 4.61 (m, 1H, **H-8**), 3.59 (dd, J_2= 12.2 Hz, J_3= 6.0 Hz, 1H, **H-9a**), 3.77 (dd, J_2= 12.9 Hz, J_3= 3.7 Hz, 1H, **H-9b**), 3.69 (s, 3H, **H_3-10** or **10′**), 7.08 (d, J_3=1.8 Hz, 1H, **H-2′**), 6.87 (d, J_3=8.64 Hz 1H, **H-5′**), 6.92 (dd, J_3=8.46 Hz, J_3=1.8 Hz 1H, **H-6′**), 6.52 (dt, J_3=15.9 Hz, J_4=1.0 Hz, 1H, **H-7′**), 6.27 (dt, J_3=15.8 Hz, J_3=6.0 Hz, 1H, **H-8′**), 4.18 (dd, J_3=6.1 Hz, J_4=1.2 Hz, 2H, **H_2-9′**), 3.71 (s, 3H, **H_3-10′** or **10**).

δ(^{13}C, D_2O, 201 MHz) 110.75 (**C-2**), 115.48 (**C-5**), 119.59 (**C-6**), 72.43 (**C-7**), 83.21 (**C-8**), 60.49 (**C-9**), 55.43 (**C-10** or **10′**), 109.81 (**C-2′**), 115.42 (**C-5′**), 119.48 (**C-6′**), 130.09 (**C-7′**), 126.70 (**C-8′**), 61.95 (**C-9′**), 55.43 (**C-10′** or **10**).

Initial QTOF MS data implied a chemical formula of $C_{20}H_{22}O_6$, however, through the NMR structure elucidation process an extra proton shift was found correlated to the molecule of interest. It was proposed that the mass initially observed in the QTOF MS was in fact the $[M\text{-}H_2O]^-$ peak, implying a formula of $C_{20}H_{24}O_7$. Further MS studies did

find the corresponding molecular ion by both +ve ESI (M^+, *m/z* 376; and $[M+Na]^+$, *m/z* 399) and -ve ESI ($[M-H]^-$, *m/z* 375) with the application of a reduced nebulizer spray temperature (see section 3.4.8 below).

Initial 1D proton NMR spectra were recorded for both ***FK1*** (458 μg) and ***FK2*** (387 μg). ***FK1*** was determined to be more pure and of higher concentration, so initial structure determination was performed on this product. ^{1}H, ^{13}C-HSQC, ^{13}C-HMBC, ^{1}H-^{1}H DQF-COSY, ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H NOESY NMR spectra were then measured for ***FK1*** and structural fragments were constructed as follows. Two methoxy resonances were identified in the ^{1}H NMR spectrum at δ 3.68 (3H, s, **H-10**) and 3.70 (3H, s, **H-10′**). Two protons at δ 6.56 (1H, dt, J = 16.0, 1.0 Hz, **H-8′**) and δ 6.30 (1H, dt, J = 15.9, 6.0 Hz, **H-7′**) were identified in a *trans* double bond arrangement due to their J_3 coupling of 16.0 Hz, and were linked to a degenerate methylene group, δ 4.25 (2H, dd, J = 6.0, 0.9 Hz, **H_2-9′**), via their J_3 and J_4 couplings respectively. Proton signals at δ 4.07 (dd, J_2= 12.3 Hz, J_3= 2.8 Hz, 1H, **H-9a**) and δ 3.94 (dd, J_2= 12.3 Hz, J_3= 6.7 Hz, 1H, **H-9b**) were linked through their J_2 coupling and their shared carbon shift in the ^{13}C-HSQC, and were linked to a multiplet at δ 4.66 (m, 1H, **H-8**) via the DQF-COSY. An ABX aromatic coupling system was identified, and positioned the aromatic proton at δ 6.86 (dd, J_3= 8.1 Hz, J_3=1.9 Hz, 1H; **H-6**) *meta* from δ 6.88 (d, J_4=2.0 Hz, 1H, **H-2**) and *ortho* to δ 6.74 (d, J_3=8.1 Hz, 1H, **H-5**). Three further aromatic protons were identified and linked via the TOCSY, but needed correlations from the HSQC and HMBC to resolve Hδ 6.97, (overlapped s, 1H, **H-6′**) as *ortho* to Hδ 6.97, (overlapped s, 1H, **H-5′**), and *para* to δ 6.98 (bs, 1H, **H-2′**). The HSQC and HMBC were also used to resolve a solvent overlapped peak, Hδ 4.7 Cδ 72.54 (overlapped m, 2H, **H-7**, **C-7**) and link this resonance to Cδ 61.52 (**C-9**). The methoxy group **H_3-10** was linked to **H-2** via the NOESY spectrum and to Cδ 147.72 (**C-3**) via the HMBC, which was also linked to **H-6** and **H-5**. Methoxy **H_3-10′** was linked to **H-2′** via the NOESY spectrum and to Cδ 149.22 (**C-3′**) via the HMBC, which was further linked to **H-5′** and **H-6′**. NOEs were observed between **H-2′**, **H6′** and **H-7′**, linking these fragments, and was positioned on the aromatic ring by the HMBC correlation between **H-7′** to Cδ 119.45 (**C-6′**) and Cδ 109.78 (**C-2′**) as was **H-8′** to Cδ 131.13 (**C-1′**). Finally, HMBC correlations between **H-6**, **H-2** and **C-7** and further correlations between **H-8** and Cδ 146.94 (**C-4′**) constructed the final compound. NOEs between **H-2′**, **H-5′** and **H-8** confirmed this ether linkage.

Assignments could easily be correlated to ***FK2*** due to the similarity of the ^{13}C-HSQC resonances to those of ***FK1***. It was then proposed that ***FK1*** and ***FK2*** were stereoisomers of the same compound. Four possible stereoisomers of the above product are possible, with chiral centers at **C-7** and **C-8**, however, two of the possible isomers have already been isolated from nature and described in the literature (Lourith, Katayama et al. 2005). The assigned shifts of both ***FK1*** and ***FK2*** agree with previously published measurements for both *erythro-* and *threo*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether, respectively. However, it is not possible to determine whether one or both of the respective two enantiomers is present (*erythro-* 7S, 8S/7R, 8R; *threo-* 7R, 8S/7S, 8R) (Fig. 3.9).

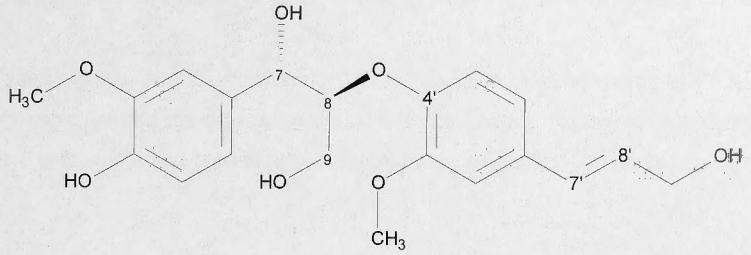
3.4.8. LC-MS/MS analysis of purified *FK1* and *FK2*

The purified neolignans, *erythro*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK1***) and *threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK2***) were subject to high resolution accurate mass analysis following electrospray ionisation (ESI) in both the negative and positive modes.

In the negative mode, the MS/MS spectra (Fig. 3.10), generated from the deprotonated ***FK1*** and ***FK2*** molecules (*m/z* 375, $[M-H]^-$) were essentially identical to those reported by Morreel et al. (2010), comprising a series of losses of H_2O (*m/z* 357, $[M-H-H_2O]^-$), formaldehyde (*m/z* 327, $[M-H-H_2O-CH_2O]^-$) and a methyl moiety (*m/z* 312, $[M-H-H_2O-CH_2O-CH_3]^-$) (Morreel, Kim et al. 2010). A comparison of the observed and calculated masses for the deprotonated molecules and their associated fragments showed them to be in good agreement (Δppm between -3.19 and 4.85) (Table 3.2).

In the positive mode, the MS spectra was dominated by the sodium adduct (*m/z* 399 ($[M+Na]^+$) and successive losses of OH (*m/z* 359, $[M-OH]^-$), H_2O (*m/z* 341, $[M-OH-H_2O]^-$) and formaldehyde (*m/z* 311, $[M-OH-H_2O-CH_2O]^-$ (Fig 3.11). A comparison of the observed and calculated masses for these ions showed them to also be in good agreement (Δppm between -1.32 and 0.58) (Table 3.3). The NMR and MS data are all consistent with ***FK1*** and ***FK2*** being identified as *erythro*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether and *threo-* guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether, respectively.

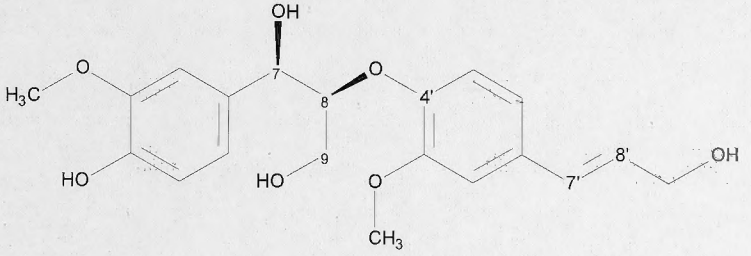
A



***Erythro*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (*FK1*)**

(7S, 8S/7R, 8R)

B



***Threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (*FK2*)**

(7R, 8S/7S, 8R)

Figure 3.9. Chemical structures of *FK1* and *FK2* as determined by NMR.

FK1 and ***FK2*** are 20 carbon molecules with two chiral carbons ($C_{20}H_{24}O_6$). Later, commercial ***FK1*** (A) was purchased from BOC Sciences, NY, USA and naturally derived ***FK2*** (B) was provided by the Second Military Medical University, Shanghai, China to confirm the structural identification and study the cellular mode of action.

A commercially purchased sample of ***FK1*** and an independently derived natural product isolate of ***FK2*** (isolated from *Bretschneidera sinensis*) were found to have essentially the same spectroscopic and chromatographic properties as the isolated ***FK1*** and ***FK2*** and were also found to promote angiogenesis in the *in vitro* rat aorta bioassay.

Xylem sap was directly analysed by LC/MS and peaks corresponding to ***FK1***, ***FK2*** and ***P6*** were found at the expected retention times, indicating that the structural integrity of these compounds had been maintained throughout the purification process.

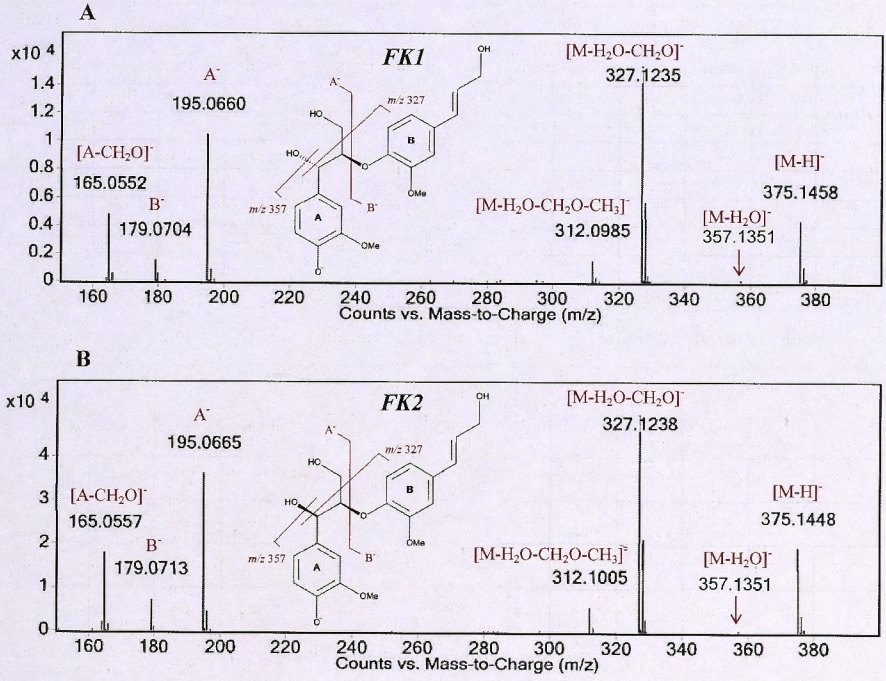
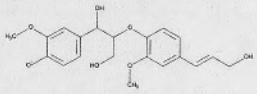
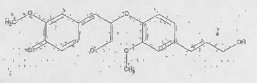
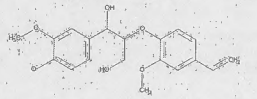
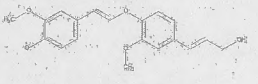
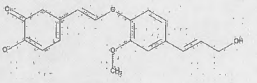
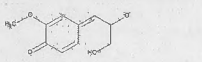
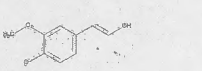


Figure 3.10. Negative ion mode ESI-MS/MS spectra.

ESI-MS/MS of ***FK1***, *erythro*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (A) and ***FK2***, *threo*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (B), selecting *m/z* 375 ([M-H]$^-$) for CID at 10eV.

Table 3.2. Comparison of elemental formulas derived from the observed negative ion mode ESI MS/MS spectra with the calculated elemental formulas for *FK1* and *FK2*.

Product ion	Structure	Empirical formula	Calc *m/z*		*m/z*	Δppm	Loss
$[M-H]^-$		$C_{20}H_{23}O_7$	375.1449	***FK1***	375.1444	1.4	H
				FK2	375.1447	0.6	
$[M-H_2O]^-$		$C_{20}H_{21}O_6$	357.1344	***FK1***	357.1355	-3.19	H_2O
				FK2	357.1351	-2.07	
$[M-CH_2O]^-$		$C_{19}H_{21}O_6$	345.1344	***FK1***	345.1333	3.08	CH_2O
				FK2	345.1347	-0.98	
$[M-H_2O-CH_2O]^-$		$C_{19}H_{19}O_5$	327.1238	***FK1***	327.1236	0.6	CH_4O_2
				FK2	327.1237	0.3	
$[M-H_2O-CH_2O-CH_3]^-$		$C_{18}H_{15}O_5$	312.1003	***FK1***	312.0989	4.56	$C_2H_7O_2$
				FK2	312.1005	-0.57	
A^-		$C_{10}H_{11}O_4$	195.0663	***FK1***	195.0663	0.09	$C_{10}H_{12}O_3$
				FK2	195.0662	0.42	
$[A-CH_2O]^-$		$C_9H_9O_3$	165.0557	***FK1***	165.0559	-1.1	$C_{11}H_{14}O_4$
				FK2	165.0560	-1.71	
B^-		$C_{10}H_{11}O_3$	179.0714	***FK1***	179.0705	4.85	$C_{10}H_{12}O_4$
				FK2	179.0713	0.38	

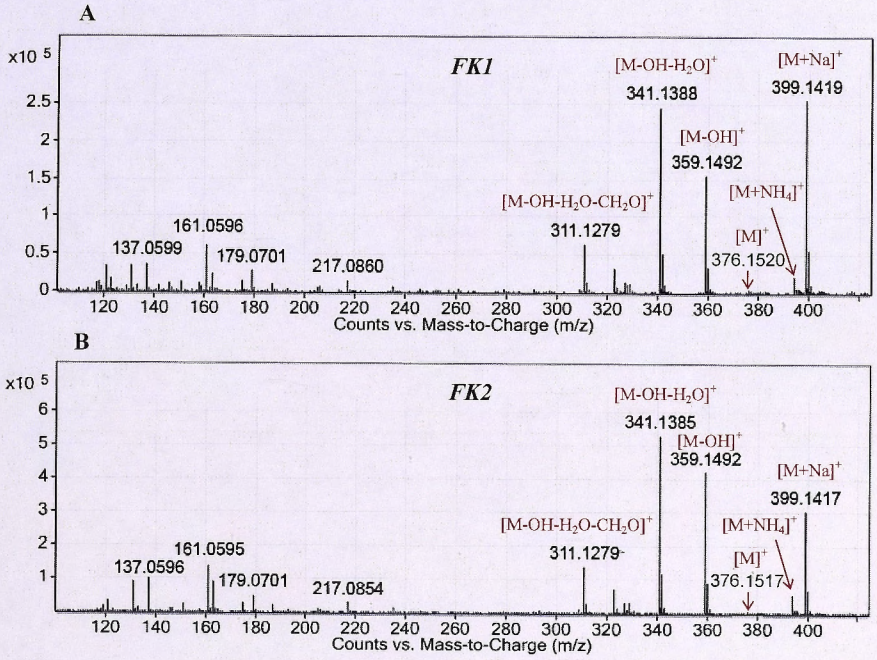


Figure 3.11. Positive mode ESI MS spectra.

Positive mode ESI-MS of ***FK1***, *erythro*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (A) and ***FK2***, *threo*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (B).

Table 3.3. Comparison of elemental formulas derived from the observed positive ESI MS/MS spectra with the calculated elemental formulas for *FK1* and *FK2*.

Product ion	Empirical formula	Calc *m/z*		*m/z*	Δppm	Gain/Loss
$[M+Na]^+$	$C_{20}H_{24}O_7Na$	399.1414	***FK1***	399.1419	-1.19	Na adduct
			FK2	399.1417	-0.69	
$[M+NH_4]^+$	$C_{20}H_{28}O_7N$	394.1860	***FK1***	394.1861	-0.18	NH_4 adduct
			FK2	394.1858	0.58	
$[M]^+$	$C_{20}H_{24}O_7$	376.1517	***FK1***	376.1520	-0.92	-
			FK2	376.1517	-0.12	
$[M-OH]^+$	$C_{20}H_{22}O_6$	359.1489	***FK1***	359.1492	-0.79	-OH
			FK2	359.1492	-0.79	
$[M-OH-H_2O]^+$	$C_{20}H_{20}O_5$	341.1384	***FK1***	341.1388	-1.32	$-H_3O_2$
			FK2	341.1385	-0.44	
$[M-OH-H_2O-CH_2O]^+$	$C_{19}H_{19}O_4$	311.1278	***FK1***	311.1279	-0.37	$-CH_5O_3$
			FK2	311.1279	-0.37	

3.5. Discussion

Historically, plants have been a source of therapeutics. Over one hundred discovered chemical substances being considered as important drugs have been derived from different plants as a result of chemical studies focused on the isolation of active compounds from plants used in traditional medicine (Cragg and Newman 2001; Cragg and Newman 2001). In plants, soybean has long been identified as a source of isoflavones, genistein and daidzein. These molecules have been associated with health benefits such as relief of menopausal symptoms (Nestel, Pomeroy et al. 1999; Murkies, Lombard et al. 2008), reduction of osteoporosis (Civitelli 1997; Gennari, Agnusdei et al. 1998), lowering blood cholesterol levels (Kirk, Sutherland et al. 1998; Merz-Demlow, Duncan et al. 2000), and reduction in risk of certain hormone related cancers i.e. breast (Peterson and Barnes 1991; Banerjee, Li et al. 2008) and coronary heart disease (Middleton, Kandaswami et al. 2000). Therefore, soybean derived flavonoids became a matter of additional interest in this project for their potential therapeutic applications.

Soybean growth studies showed that the yield of pro-angiogenic compounds could be maximised when uninoculated plants, planted in early to mid-summer, were grown in the presence of 10 mM KNO_3. The enriched xylem sap from these plants could then be quickly passed through the spin columns to yield an activity-containing, low molecular weight fraction, as determined by the *in vitro* rat aorta bioassay. Bioactivity guided fractionation showed three consistent areas of pro-angiogenic activity in both *Bradyrhizobium* inoculated and 10 mM KNO_3 treated soybean extracts (Fig. 3.3). Initially, ***FK1***, ***FK2*** and ***P6*** were purified for structural determination. However, ***P6*** appeared to be very low in quantities in different plant cultures, seasonal dependent and also unstable. Therefore, ***FK1*** and ***FK2*** were chosen for structural elucidation and further experiments.

FK1 and ***FK2*** were then purified by semi-preparative HPLC to yield sufficient material (~ 400 μg) for structural elucidation using NMR and high resolution accurate mass spectra, enabling their identification as *erythro*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK1***), and *threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK2***). In plants, these molecules are derived from the dimerisation of monolignols which are the precursors of the lignin, a key structural element laid down in the secondary plant cell

walls. There are three monolignols, p-coumaryl, coniferyl and synapyl alcohols which can be dimerised and polymerised into lignans and the lignin complex, respectively (Fig. 3.12) (Whetten and Sederoff 1995; Boerjan, Ralph et al. 2003).

Lignin, the second most abundant polymer after cellulose, is an aromatic heteropolymer found mainly in secondary-thickened plant cell walls and xylem cells (Whetten and Sederoff 1995). It provides strength for the plant to grow upwardly and also allows the transport of water, solutes and nutrients through the vascular system along with protecting plants from pathogen infection and other environmental stresses (Whetten and Sederoff 1995; Boerjan, Ralph et al. 2003). However, in angiosperms (flowering plants), lignin polymer is made predominantly from the monolignols coniferyl and sinapyl alcohol (Fig. 3.12) (Baucher, Monties et al. 1998), while the lignin of gymnosperms (seed-bearing plants) does not have sinapyl alcohol precursor (Morreel, Ralph et al. 2004).

Lignans and neolignans are phenylpropanoid dimers with a wide range of structural diversity (Mander and Liu 2010) which have previously been shown to have a number of medically important biological activities such as anti-tumour (Qu, Madl et al. 2005; Zhou, Liu et al. 2009), anti-oxidant (Kancheva, Saso et al. 2012) and anti-inflammation activities (Hallund, Tetens et al. 2008; Baumgartner, Sosa et al. 2011).

In angiosperms (soybean) the monolignols coniferyl and sinapyl alcohol are transported to the cell wall where they are dehydrogenised by oxidative enzymes, such as peroxidases or laccases to form radicals with unpaired electron density at positions including *O*-C4-, C5-, and C8- (Boerjan, Ralph et al. 2003; Damiani, Morreel et al. 2005; Morreel, Dima et al. 2010). The radicals then bind by a combinational radical-radical coupling mechanism to form C8-C8′, C5-C5′, C8-C5′, C4-*O*-C5′ and C8-*O*-C4′ dimers (Fig. 3.13) (Bunzel, Ralph et al. 2001; Peterson, Dwyer et al. 2010). However, by definition, only C8-C8′ are known as lignans and the other dimers (C5-C5′, C8-C5′, C4-*O*-C5′ and C8-*O*-C4′) are classified as neolignans (Macrae and Towers 1984). Clearly, some of these dimers either leak out into the xylem sap, albeit in low concentrations, or are transported in xylem sap prior to being polymerised into high molecular weight lignins. Back calculations from the HPLC profile showed the concentrations of ***FK1*** and ***FK2*** to be approximately 5×10^{-7} M in concentrated soybean xylem sap.

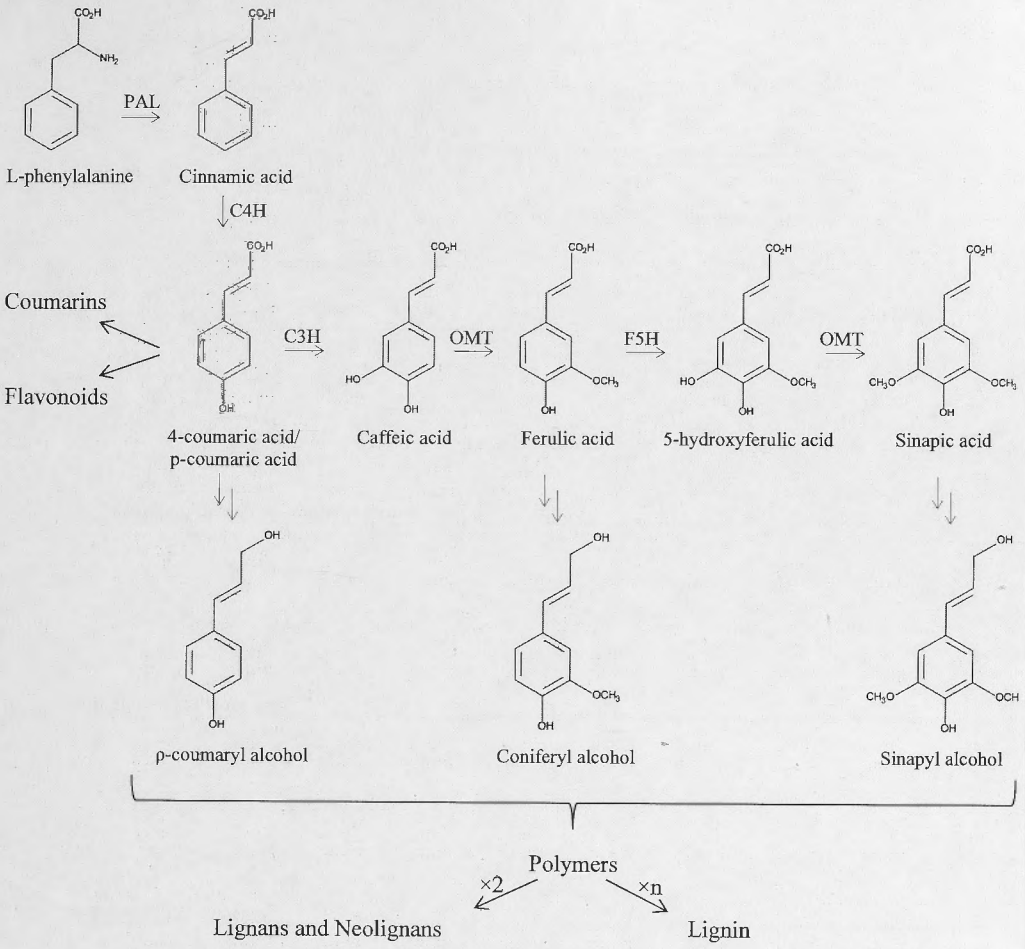
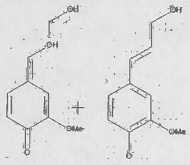
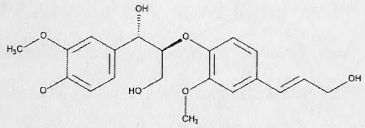


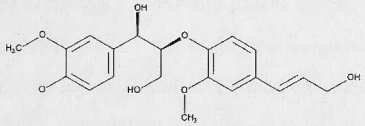
Figure 3.12. An overview of monolignol biosynthetic pathway as precursors for lignans, neolignans and lignin.

L-Phenylalanine which is produced by the shikimic acid pathway is de-aminated by phenylalanine ammonia-lyse (PAL) to yield cinnamate. Cinnamate is hydroxylated by cinnamate 4-hydroxylase (C4H) to form ρ-coumarate. The hydroxyl groups can be methylated by an *O*-methyltransferase (OMT). Ferulic acid is hydroxylated by ferulate 5-hydroxylase (F5H). The ρ-coumaroyl-CoA, feruloyl-CoA and sinapol-CoA intermediates give rise to ρ-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively following the action of cinnamyl alcohol dehydrogenase (CAD) on aldehyde forms. These monolignols can then be dimerised to form lignans and neolignans such as ***FK1*** and ***FK2*** or polymerised to yield lignin. Adapted from (Whetten and Sederoff 1995; Boerjan, Ralph et al. 2003).

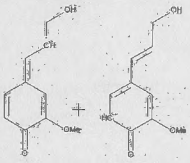


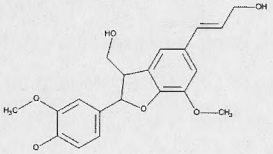


G(e8-O-4)G, *erythro*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK1***)

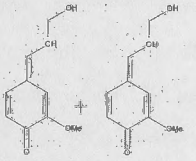


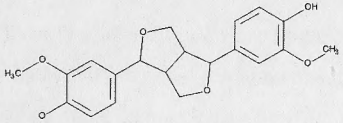
8-*O*-4′ coupling, β-ether G(t8-O-4)G, *threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK2***)



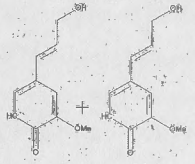


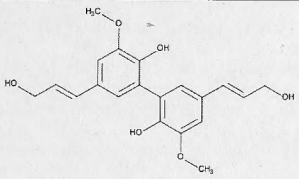
8-5′ coupling, Dehydrodiconiferyl alcohol



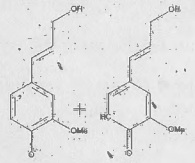


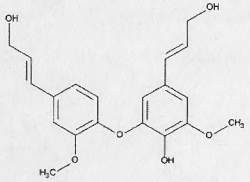
8-8′ coupling, Pinoresinol





5-5′ coupling, Biphenyl





4-*O*-5′ coupling, biphenyl ether

Figure 3.13. Radical-radical coupling of monolignols (Coniferyl alcohol).

Monolignol radicals can covalently bind to form C8-C8′ (lignan), C5-C5′, C8-C5′, C4-*O*-C5′ and C8-*O*-C4′ (neolignan) dimers. However, C8-*O*-C4′, C8-C5′ and C8-C8′ dimers are main types of monolignol dimerisations. Adapted from (Boerjan, Ralph et al. 2003; Morreel, Ralph et al. 2004; Morreel, Dima et al. 2010).

Both of these 8-*O*-4′ neolignans (***FK1*** and ***FK2***), were found to be obtained commercially (in the case of ***FK1***) or available in collections of natural products. ***FK2*** was obtained from a collection of 6000 purified natural products from the Laboratory of Natural Products in the School of Pharmacy of the Second Military Medical University in Shanghai, China. These samples were confirmed as having identical spectroscopic (Morreel, Dima et al. 2010; Morreel, Kim et al. 2010) and biological properties as the biologically derived material, opening the way for extensive study of the mode of action on human model cell lines (Chapter 4).

For reasons that were not clear, the yield of ***P6*** from the plants proved to be variable and P6 was also found to be unstable to long term storage at -20 °C. By contrast, ***FK1*** and ***FK2*** proved to be stable to repeated freeze/thaw cycles and could be satisfactorily stored at -20 °C for periods in excess of a year.

Overall, we were able to successfully identify and purify two pro-angiogenic small molecules from *Glycine max* xylem sap by bioactivity directed fractionation using the *in vitro* rat aorta bioassay. These two molecules were then found to be from the lignan family and were identified as *erythro*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (***FK1***) and *threo*-guaiacylglycerol-8-*O*-4′-(coniferyl alcohol) ether (***FK2***) using NMR and MS data. Independently-derived samples of these two neolignans were found to have essentially the same spectroscopic and chromatographic properties as the isolated ***FK1*** and ***FK2*** and were also found to promote angiogenesis in the *in vitro* rat aorta bioassay. To our knowledge, this is the first report on angiogenic activity of these molecules. The cellular mode of action studies on their mechanism of promoting angiogenesis will be further studied in chapter 4 using the independently-derived counterparts of ***FK1*** and ***FK2***.

Chapter 4

The Cellular Mode of Action Studies of *Erythro*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (*FK1*) and *Threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (*FK2*)

4.1. Introduction

Angiogenesis is the formation of new capillaries from a pre-existing vascular network. Angiogenesis is a tightly controlled process in adult mammals (Fig. 1.5) and many pathological states associated with major classes of chronic diseases are associated with either excessive or insufficient angiogenesis. For example, excessive angiogenesis is associated with diseases such as cancer, psoriasis, blindness induced by age-related macular degeneration (AMD) and arthritis (Folkman 2000; Carmeliet 2003; Folkman 2007). Therefore, there has been much interest in the discovery of anti-angiogenic agents and a diverse and extensive range of anti-angiogenic molecules have been isolated and identified and tested in clinical trials (Pezzuto 1997; Newman 2011).

More recently, the possibility of using pro-angiogenic molecules as therapeutic agents has emerged. Pro-angiogenic drugs are less researched than anti-angiogenic drugs nonetheless they also have potential to aid in the treatment of a range of clinical situations (Carmeliet 2003) where the formation of new blood vessels is needed, such as in wound healing (Tonnesen, Feng et al. 2000; Bao, Kodra et al. 2009), cardiovascular disease (Pandya, Dhalla et al. 2006) and ischemic conditions such as stroke (Beck and Plate 2009; Font, Arboix et al. 2010).

Moreover, only a limited number of pro-angiogenic molecules have been studied in clinical settings. These have included erythropoietin (EPO) (Ribatti, Presta et al. 1999), β-carotene (Dembinska-Kiec, Polus et al. 2004), nicotine (Mousa and Mousa 2006; Morimoto, Takemoto et al. 2008; Costa and Soares 2009), ferulic acid (Lin, Chiu et al. 2010) and stromal cell-derived factor 1 (SDF-1) (Deshane, Chen et al. 2007). Therefore, it is of great interest to identify and explore more compounds with pro-angiogenic activity.

Plants have been used for centuries as a source of therapeutics to treat disease and continue to be the source of many new potential therapeutic agents. In more recent times, plant extracts have been screened using bioassays to discover the active ingredients of plants used in traditional medicine (Balunas and Kinghorn 2005). In chapter 3, two small molecules (***FK1*** and ***FK2***) were isolated from soluble fractions of soybean and shown to enhance angiogenesis using *in vitro* mammalian angiogenesis assays. The structures of ***FK1*** and ***FK2*** were then determined using NMR and high

resolution accurate mass spectroscopy, to be *erythro*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK1***) and *threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK2***) (Fig. 3.9).

In plants, ***FK1*** and ***FK2*** are precursors for lignin formation (Boerjan, Ralph et al. 2003; Morreel, Dima et al. 2010). Next to cellulose, lignin is the most abundant macromolecule on earth and it provides the structural support to enable woody higher plants to support their own weight, grow to great heights and resist wind damage. Molecules such as ***FK1*** and ***FK2*** are examples of structurally-diverse plant secondary metabolites derived from phenylpropanoid metabolism which are cross linked randomly in free radical-dependent reactions to form lignins. ***FK1*** and ***FK2*** are formed primarily from monolignols, coniferyl alcohol, by dehydrogenative dimerisation and distinguished by the intermonomer C8-*O*-C4′ linkage (Fig. 3.13). From this family, dimers from monolignols (ρ-coumaryl, coniferyl and synapyl alcohols) with C8-C5′ and C8-C8′ bonds have been previously reported as having bioactivity in humans (Milder, Feskens et al. 2005; Van Miert, Van Dyck et al. 2005; Peterson, Dwyer et al. 2010). Therefore, molecules derived from the same plant pathway may have multiple activities and may represent a rich treasure trove of potential therapeutics.

This chapter describes detailed studies of the angiogenesis modulating activity of synthetic ***FK1*** and an independently derived natural source of ***FK2*** (isolated from *Bretschneidera sinensis*). Commercial ***FK1*** was purchased from BOC Sciences, NY, USA and the naturally derived ***FK2*** was provided as a gift from Prof. Dr. Wei-Dong Zhang and Dr. Shan Lei from the Second Military Medical University, Shanghai, China. These molecules were shown to exhibit pro-angiogenic activity based on angiogenesis bioassay studies on their naturally occurring counterparts isolated from soybean (in chapter 3). Since molecules such as ***FK1*** and ***FK2*** occur throughout the plant kingdom, they are likely to be a common component of the human diet. Therefore, ***FK1*** and ***FK2*** might be expected to have low toxicity. Given this, this increases the likelihood that they could potentially be developed as new drugs for manipulating angiogenesis. In this chapter, ***FK1*** and ***FK2*** were tested in several *in vitro* angiogenesis assays, which represent some of the key steps involved in angiogenesis. By testing each of these steps individually this offered the possibility of dissecting angiogenesis and perhaps pinpointing which step in this complex and highly regulated process could be targeted by ***FK1*** and ***FK2***. By exploring these research avenues, important information about the cellular mode of action of these molecules could be determined. The bioassays were

aimed at determining if ***FK1*** and ***FK2*** affected endothelial cell proliferation, migration, tube formation or their adhesion to endothelial cell ECM components.

4.1.1. Endothelial cell proliferation assay

Endothelial cell proliferation is one of the important steps in angiogenesis and occurs in response to a number of angiogenic factors to provide sufficient cells to form new vessels. Pro-angiogenic or anti-angiogenic effects of test compounds on cell proliferation can be measured by direct cell counts, quantification of DNA synthesis, or assessment of metabolic activity (Goodwin 2007).

Among the well-established cell proliferation assays, thymidine incorporation is the most frequently used assay. The ^{3}H-thymidine incorporation assay has been carried out widely in order to identify if test substances can either enhance or inhibit angiogenesis by influencing DNA synthesis (Auerbach, Lewis et al. 2003). Proliferation assays can be carried out with low- or serum-free conditions to measure the pro-angiogenic activity of test compounds. Growth factors can be also applied to the serum-free culture medium. Anti-angiogenic activity of the compounds of interest is measured using normal levels of serum and/or growth factors (Goodwin 2007).

In this thesis, the proliferation of human umbilical vein endothelial cells (HUVEC) was followed in the presence of ***FK1*** and ***FK2*** in serum free medium. Basic fibroblast growth factor (bFGF) was added in some experiments to evaluate if the compounds influence the mitogenic effects of bFGF.

4.1.2. Endothelial cell tube formation assay

Angiogenesis requires the assembly of endothelial cells into vessel tubes. One of the most specific tests for angiogenesis modulators is to screen for their ability to affect endothelial cells forming three-dimensional tubular structures on an appropriate basement membrane matrix (Auerbach, Lewis et al. 2003; Goodwin 2007; Arnaoutova and Kleinman 2010). *In vitro* bioassays measuring tube formation involve several steps of new blood vessel formation, including endothelial cell migration, adhesion to extracellular matrix components, and differentiation (Grant, Lelkes et al. 1991; Arnaoutova and Kleinman 2010). Therefore, the endothelial tube formation assay

usually will be followed with endothelial migration and adhesion assays as they are mostly focused on a single specific step in angiogenesis (Conn 2012). Matrigel is the most widely used basement membrane model along with fibrin and collagen clots. It is an artificial ECM derived from Engelbreth-Holm-Swarm (EHS) tumour cells and highly enriched in laminin (a predominant extra cellular matrix component), collagen IV, HSPG and various growth factors that initiate the formation of tubules (Auerbach, Lewis et al. 2003; Arnaoutova and Kleinman 2010; Conn 2012). Endothelial cell tube formation on Matrigel is a very quick assay and can be assessed within 24 hours. Initial vascular networks become evident after 2 hours of seeding endothelial cells onto the basement membrane and tubular bodies form after 5 hours. The capillary like structures become complete in 20 hours with the contraction of cell aggregates and then the fusing and elongation of cell branches (Conn 2012). In the current work, the effect of ***FK1*** on endothelial cell tube formation on Matrigel was measured using HUVEC and HMEC cultures.

4.1.3. Endothelial cell migration assay

During angiogenesis, endothelial cells migrate into surrounding tissue following extra cellular ECM degradation. Endothelial cell migration occurs in response to a gradient of angiogenic stimulatory factors, inducing VEGF and FGFs (Turner and Grose 2010; Weis and Cheresh 2011). Therefore, endothelial cell migration is an essential component of angiogenesis that requires a tight regulation of signalling and physiological mechanisms (Lamalice, Le Boeuf et al. 2007).

In vitro cell migration assays can be used to determine the cell motility response to a particular angiogenic modulating agent. These assays include trans-well (based on modified Boyden chambers), under-agarose and scrape-wound assays (Auerbach, Lewis et al. 2003; Goodwin 2007; Staton, Reed et al. 2009).

In the scrape-wound assay, a wound is introduced to the monolayer of confluent endothelial cells using a pipette tip, needle or cell scraper. The speed of cell migration towards the cleared area can be assessed over time by measuring the distance moved by endothelial cells as the wound closes. Closing the wound in this assay is initially based on cell migration, although cells in the cleared area will eventually proliferate as well. Therefore, for pro-angiogenic compounds enhancing migration, rather than cell

proliferation, can be addressed in the scrape wound assay by adding anti-proliferative agents to the culture medium (Goodwin 2007). In this project, endothelial cell migration was evaluated using HMEC monolayer and the IncuCyte wound scraper and imaging system.

4.1.4. Endothelial cell adhesion to ECM components

Endothelial cell adhesion to the ECM plays an essential role in angiogenesis and it is mediated by adhesion molecules such as integrins. Integrins are cell-matrix adhesion molecules that bind to ECM components and integrate signals between the ECM and intracellular pathways (Weis and Cheresh 2011). During angiogenesis, the expression of angiogenic specific integrins that bind to fibronectin and vitronectin ECM ligands increases and this induces migration and tube formation events (Liekens, De Clercq et al. 2001; Weis and Cheresh 2011).

Over time, various methods have been used for *in vitro* adhesion assays to quantify the binding of cells to the target molecules. These techniques include counting cells under a microscope (Lackie and Bono 1977) using a particle counter (Mcfaul and Bowman 1990) and radiolabeling the cells using either ^{3}H-thymidine (Roszkowski, Beuth et al. 1989) or ^{51}Cr (Haskard, Strobel et al. 1989) and measuring the incorporated radioactivity of adherent cells. Recently cell adhesion has been estimated by staining adherent cells with dyes such as Rose Bengal and measuring the optical density (OD) of the absorbed dye within the bound cells (Chong and Parish 1985; Pan, Ren et al. 2012).

Thus, in the present work, the adhesion of HMEC to immobilised fibronectin and vitronectin, as good representatives of angiogenic ECM components, was determined using the Rose Bengal adhesion assay.

4.2. Research aims

Over the past 20 years, searches for effective anti-angiogenic therapeutics have been central to pharmacological research relating to angiogenesis. Anti-angiogenic molecules may be effective treatments for diseases such as solid tumour formation where the provision of a blood supply is thought to be important for tumour progression (Kerbel 1997). The aim of this research was to develop potential pro-angiogenic drugs that may find utility in treating several cardiovascular disorders or to aid wound healing. As a result of research conducted in chapter 3, two pro-angiogenic molecules derived from soybean were isolated. In this chapter, the validity of the biological activity of these molecules was first determined using synthetically-derived or independently sourced molecules of identical structure and the cellular mode of action of these molecules was studied using biological assays that incorporated the major steps of angiogenesis.

4.3. Research objectives

1) Confirm the pro-angiogenic activity of the synthetic versions of ***FK1*** and ***FK2*** using the rat aorta pro-angiogenesis bioassay.

2) Investigate the pro-angiogenic activity of the synthetic molecules at physiologically relevant concentrations.

3) Investigate the cellular mechanism of action of ***FK1*** and ***FK2*** in enhancing angiogenesis using human endothelial cells in specific angiogenesis bioassays.

4) Determine their possible interactions with angiogenic growth factors such as bFGF and its receptor.

4.4. Results

4.4.1. *In vitro* activity of synthetic *FK1* and independent naturally-derived *FK2* in the rat aorta ring bioassay

Initially, standard rat aorta ring model (RARM) angiogenesis assays were performed using growth medium containing 20% Heat Inactivated FCS (HIFCS). The level of growth factors in HIFCS at this concentration allows vessels to grow at near maximum capacity. This results in newly forming vessels occupying approximately 80-90% of the observable field of view after 7 days of culture as they encroach on the walls of the containment vessel (48-well plate). The standard assay was configured to measure the inhibition of angiogenesis at a day 5 endpoint by manually quantifying the relative density and number of new vessels sprouted from the aorta ring in the field of view at this time.

In this chapter, the standard RARM assay was modified to maximise the probability of identifying compounds with pro-angiogenic activity. This involved performing the pro-angiogenesis bioassay using 5% HIFCS instead of 20% HIFCS. This resulted in slower vessel outgrowth (approximately 60% vessel occupancy occurred after 7 days of culture). It also enabled the stimulatory and inhibitory effects of test compounds to be measured manually based on field of view observation on three days (days 5-7) instead of one day (Fig. 4.1). Successful utilisation of this modified human angiogenesis assays was first used to determine the pro- or anti-angiogenic activity of Nod factors (Susanti 2011; Djordjevic, Bezos et al. 2013) as well as screening for compounds from soybean that could either enhance or inhibit vessel outgrowths (Du Fall 2009). This modified assay was also used in this thesis.

Independently derived versions of ***FK1*** and ***FK2*** were either purchased (i.e. synthetically derived; ***FK1***) or obtained as a purified chemical from a plant extract (***FK2***). The biological activity of these ***FK1*** and ***FK2*** preparations was investigated over a range of concentrations (5×10^{-6} M to 5×10^{-9} M) to independently confirm that they had the same activity as those derived from soybean, and to satisfy Koch's molecular postulates (Falkow 1988). The results, expressed as percent growth, showed significant enhancement of vessel outgrowth compared to the control after 5, 6 and 7 days of culture at all concentrations examined (Fig. 4.2). Since the independent samples of

synthetic ***FK1*** and naturally derived ***FK2*** reflected the activity of soybean derived ***FK1*** and ***FK2***, they were then used for the cellular mode of action studies outlined in this chapter.

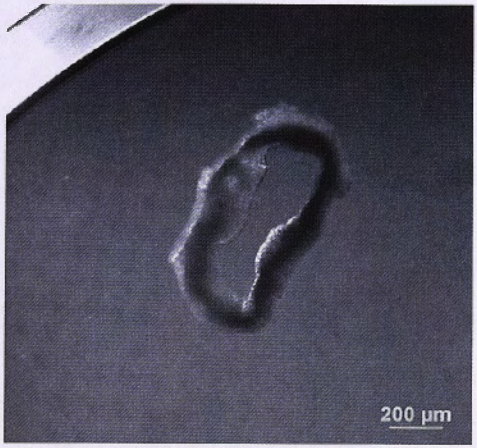
4.4.2. Effects of synthetic *FK1* and independent naturally-derived *FK2* on endothelial cell proliferation

Synthetic ***FK1*** and naturally derived ***FK2*** were tested from 5×10^{-6} M to 5×10^{-12} M for their effect on the proliferation of serum-starved confluent HUVEC with or without bFGF at 12.5 ng/mL (Fig. 4.3). Previously, bFGF had been used at 25 ng/mL (Susanti 2011) to test for the activity of Nod factor derivatives. However, the pro-angiogenic activity of ***FK1*** and ***FK2*** was considerably higher than the Nod factor derivatives (Susanti 2011). Thus, bFGF was used at 12.5 ng/mL instead to assess the mitogenic effects of ***FK1*** and ***FK2*** in the presence and absence of bFGF.

Synthetic ***FK1*** and naturally derived ***FK2*** significantly induced HUVEC proliferation at 5×10^{-6} M to 5×10^{-8} M and 5×10^{-6} M to 5×10^{-12} M concentrations, respectively, when cultures contained 12.5 ng/mL of bFGF. The peak of activity occurred at 5×10^{-8} M in both cases and tapered off at lower and higher concentrations. In addition, the results showed that in the absence of bFGF, ***FK2*** (but not ***FK1***) significantly enhanced HUVEC proliferation between 5×10^{-8} M to 5×10^{-10} M compared to the control.

These results suggest that both ***FK1*** and ***FK2*** require bFGF to significantly enhance cell proliferation but ***FK2*** may also induce proliferation in the absence of bFGF.

A



B

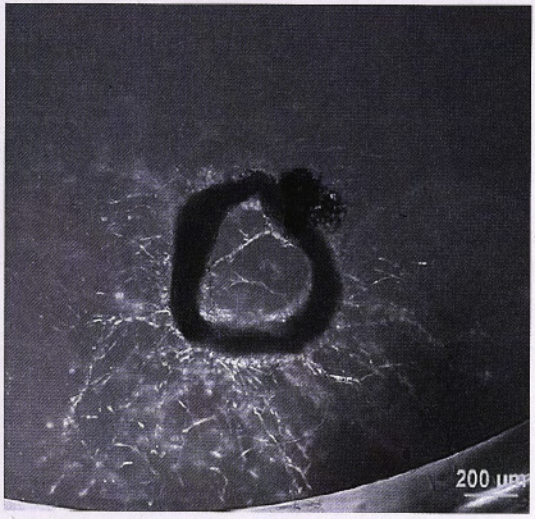


Figure 4.1. Measuring the activity of the test compounds in *in vitro* rat aorta angiogenesis assay.

In this thesis, the pro-angiogenic activity of the test compounds is expressed as a percentage of aorta vessel outgrowths observed on day 5, 6 and 7 based on the field view. 0% outgrowth (A), 60% outgrowth (B).

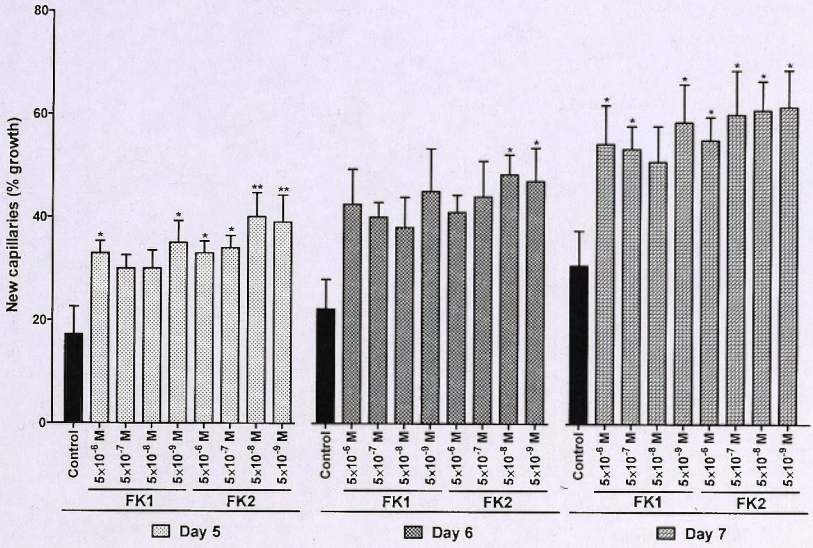


Figure 4.2. Pro-angiogenic activity of synthetic *FK1* and independent naturally-derived *FK2* in the *in vitro* rat aorta assay.

Synthetic ***FK1*** and independent naturally-derived ***FK2*** were tested at concentrations from 5×10^{-6} M to 5×10^{-9} M. Control cultures contained the same diluent dilution as the test compounds. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5, 6 and 7. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01.

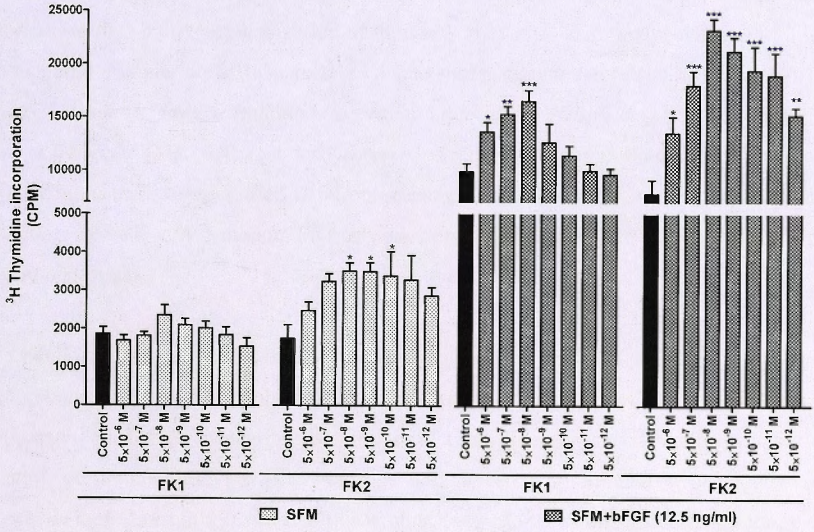


Figure 4.3. HUVEC proliferation assay of synthetic *FK1* and independent naturally-derived *FK2*.

Effect of synthetic ***FK1*** and independent naturally-derived ***FK2***, at concentrations from 5×10^{-6} M to 5×10^{-12} M on confluent serum-starved HUVEC proliferation in serum free media (SFM) with and without the mitogen bFGF (12.5 ng/mL). Control cultures contained the same diluent dilution as the test compounds. Cell proliferation was measured as ^{3}H thymidine incorporation after 24 hours incubation. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01, ***, ≤ 0.001. A two-way ANOVA data analysis showed significant difference for all tested concentrations comparing their effect in SFM to SFM+bFGF (12.5 ng/mL) treatments, $P \leq 0.0001$.

4.4.3. Effect of synthetic *FK1* on endothelial cell differentiation (tube formation assay)

To determine the effect of synthetic ***FK1*** on endothelial cell tube formation, *in vitro* HUVEC and HMEC tube formation assays on a Matrigel plug were employed. HUVEC (4×10^4 cells/well) were cultured on Matrigel in 96 well plates and incubated at 37 °C in an IncuCyte incubator and observations were recorded every 2 hours. The tube formation process includes the following steps: migration and initial tube formation (0-2 hour), reorganization of tubular bodies into larger polygonal shapes (2-6 hour), and completion of tube formation into more angular polygonal structures (6-18 hour) (Fig. 4.4). Tube formation was evident after 2 hour culture, and became complete in 12-18 hour (Fig. 4.4). It was found that after 18 hour culture, cells lost their connections and started to die. Different steps in tube formation in control cultures with cultures treated with synthetic ***FK1*** at concentrations from 5×10^{-6} M to 5×10^{-12} M were compared.

Semi-quantitative analyses have been established to measure the capacity of test compounds to induce or inhibit tube formation in this assay. Until recently, most approaches involved measuring the number of tubes, the number of branch points, the length of tubules, and the percentage of area covered by tubules versus total area (Aranda and Owen 2009). In this thesis, tube formation was quantified using the NIH ImageJ and IncuCyte software at earlier time points (4 hour incubation) by measuring percentage denuded areas and number of sprouting cells, and at later time points (6 hour incubation) by measuring the number of tubes and the total tube length (Fig. 4.5). The results showed that synthetic ***FK1*** significantly enhanced tube formation above control levels with all four measured parameters and activity tapered down with increasing dilution. The total number of sprouting cells and the number of tubes were significantly higher with synthetic ***FK1*** addition at the high concentrations tested (5×10^{-6} M and 5×10^{-7} M) whereas denuded areas and total tube length did not reach to statistical significant levels (Fig. 4.6).

Further experiments were performed with HMEC (4×10^4 cells/well), to investigate the tube formation response on a Matrigel plug. Tube formation was evident after 2 hour incubation and a very strong response was observed at higher concentrations (5×10^{-6} M and 5×10^{-7} M) of synthetic ***FK1*** (Fig. 4.7; shows results for 5×10^{-6} M only).

HMEC are smaller than HUVEC in size and consequently the sprouting cells and tubes are shorter. Therefore, tube formation can be analysed by number of completed circles. The HMEC tube formation experiment confirmed the stimulatory effect of synthetic ***FK1*** on HUVEC tube formation on Matrigel plugs (Fig. 4.8). Also, similar HUVEC and HMEC tube formation enhancement results suggested that HMEC is a good endothelial cell candidate for pursuing the further cellular mode of action studies.

In conclusion, ***FK1*** significantly enhanced tube formation of both HUVEC and HMEC on Matrigel and the effect was titrated out from higher to lower test concentrations (5×10^{-6} M to 5×10^{-12} M).

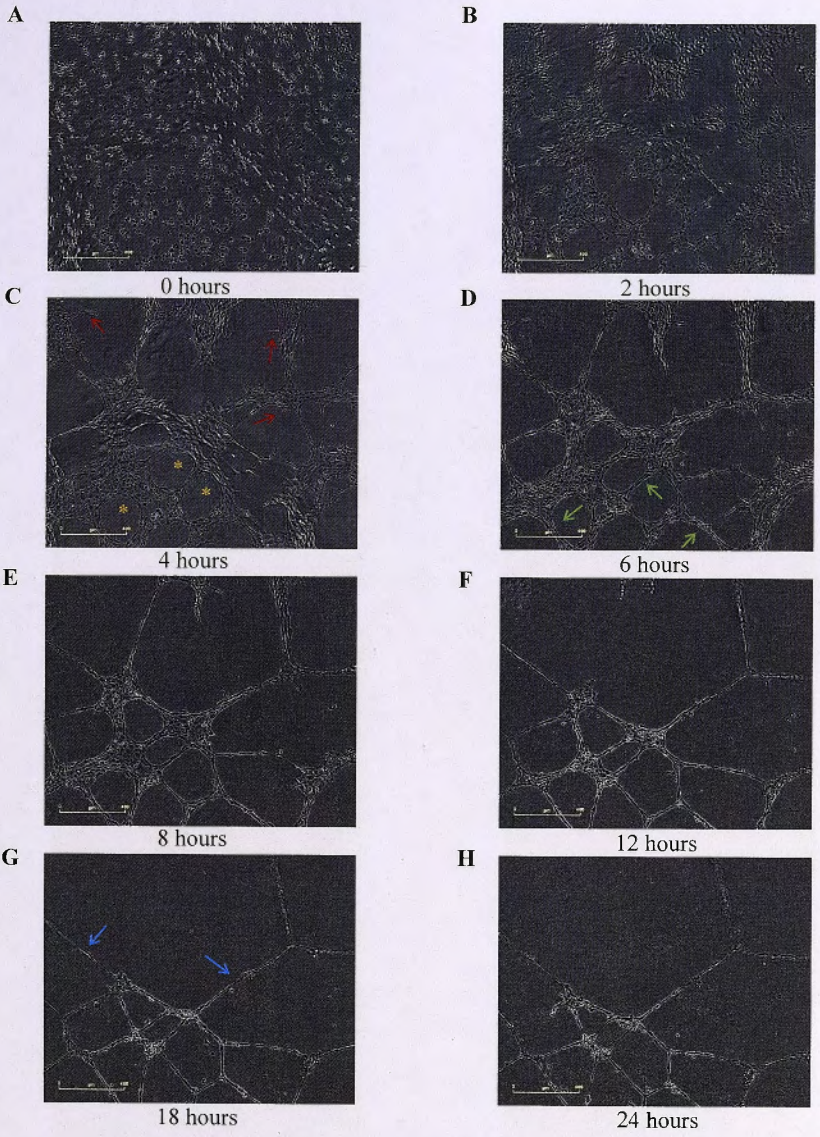


Figure 4.4. Time course and quantification of HUVEC tube formation assay on Matrigel in an untreated control.

Different stages observed when 4×10^4 HUVEC/well were seeded onto Matrigel and the same field of focus was followed for the entire 24 hour incubation period. HUVEC at 0 hours (A). Migration and initiation of formation of tubular bodies, 2 hours (B). Reorganisation of tubular bodies into larger polygonal shapes, 6 hours (C, D, E). Completion of tube formation into more angular polygonal structures, 12 hours (F). Death of cells and loss of cell connections begins to become evident, 18 and 24 hours (G, H). Denuded area (*), sprouting cell (→), tube (→), losing connections (→).

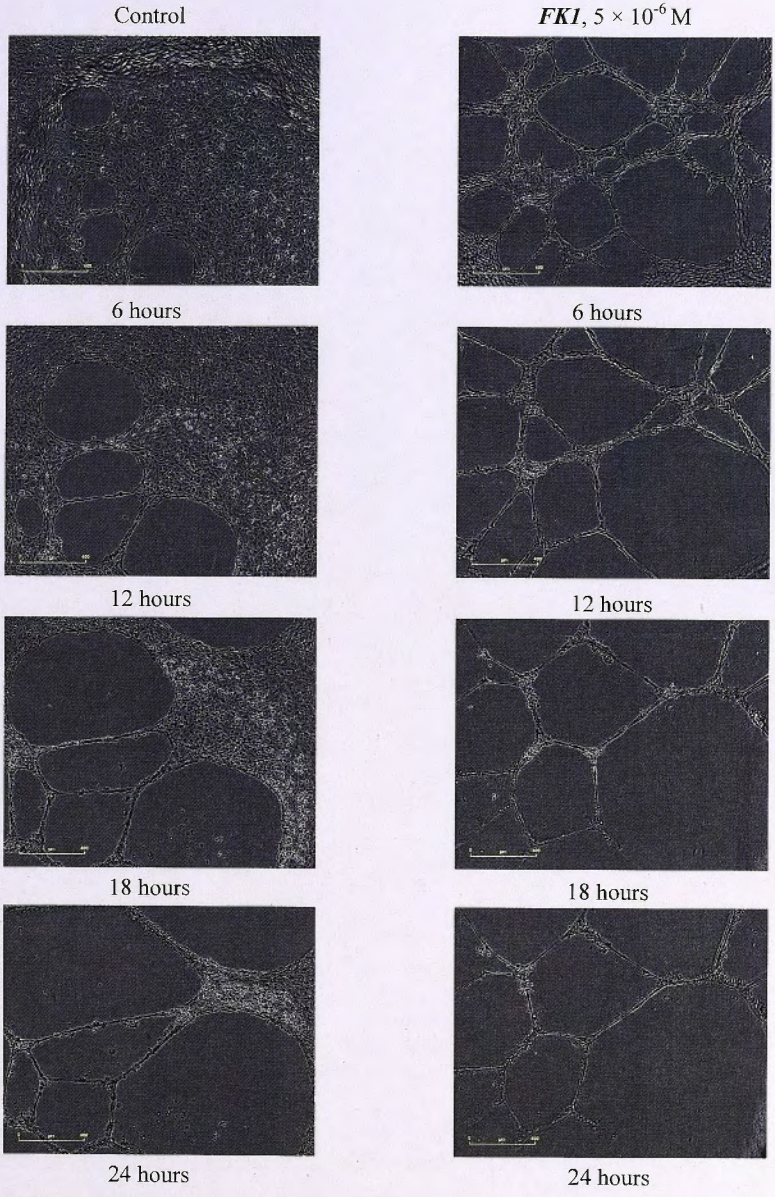


Figure 4.5. Effect of synthetic *FK1* on HUVEC tube formation assay on Matrigel.

Effect of synthetic ***FK1*** at a concentration of 5×10^{-6} M on HUVEC tube formation was observed from 0 to 24 hours. Control cultures contained the same diluent dilution as the test compounds.

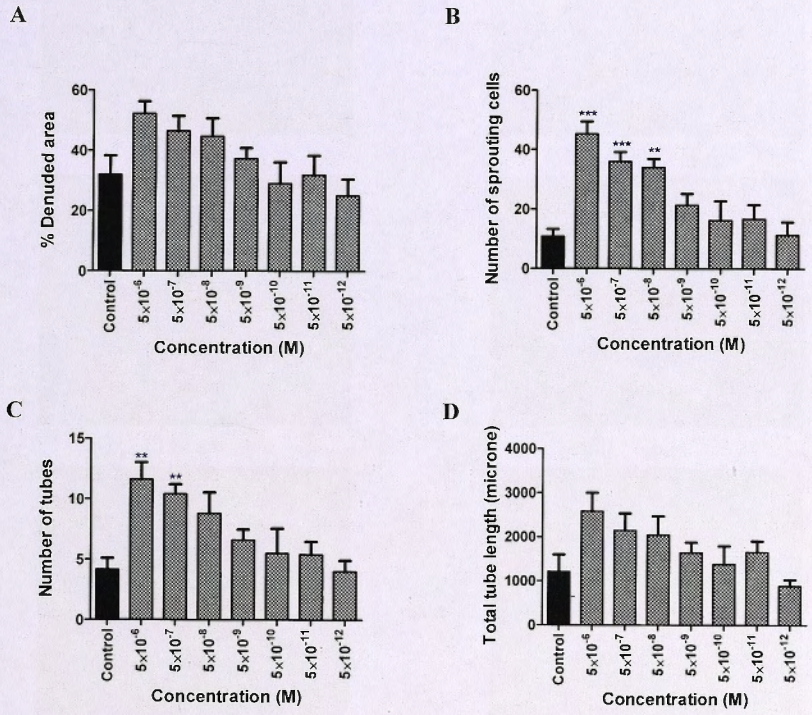


Figure 4.6. Effect of synthetic *FK1* on various parameters of a HUVEC tube formation assay on Matrigel.

Effect of synthetic ***FK1***, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on HUVEC tube formation was measured, as percentage denuded area (A), number of sprouting cells (B), number of tubes (C) and total tube length (D). Parameters shown in (A) and (B) were measured after 4 hours culture and in (C) and (D) after 6 hours culture. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). **, ≤ 0.01, ***, ≤ 0.001.

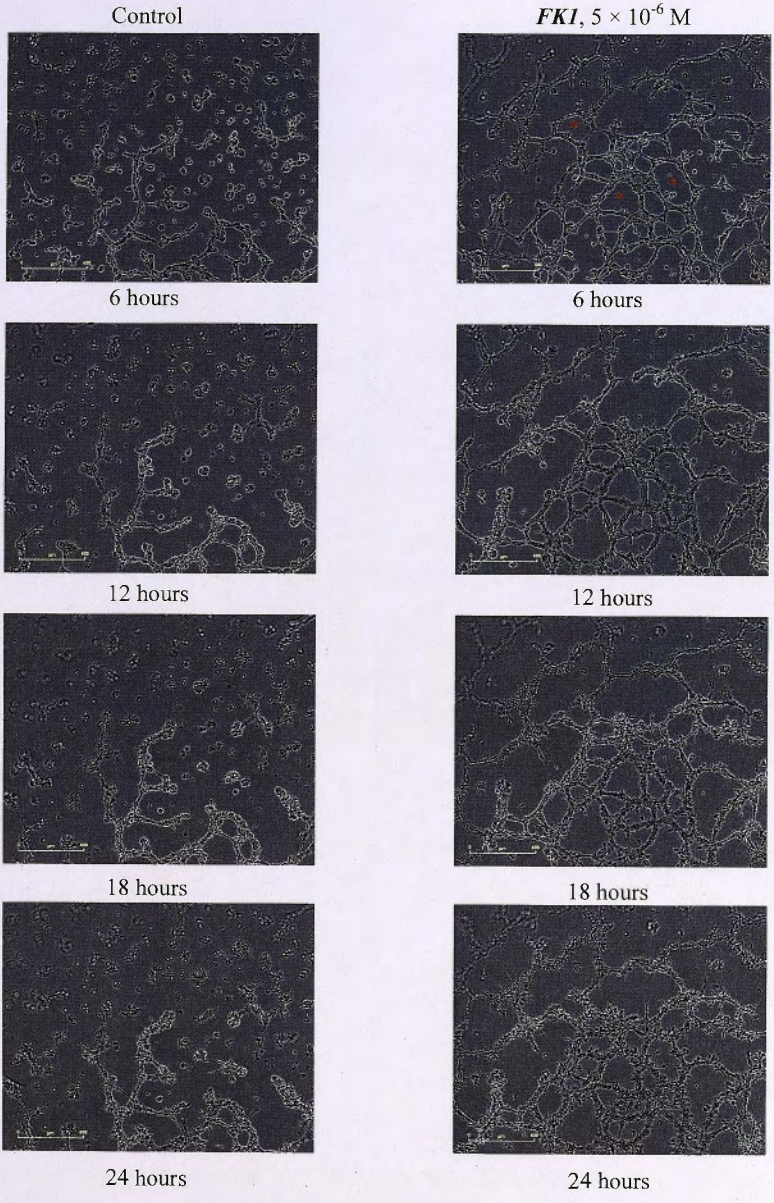


Figure 4.7. Effect of synthetic *FK1* on HMEC tube formation assay on Matrigel.

Effect of synthetic ***FK1***, at a concentration of 5×10^{-6} M, on HMEC tube formation was observed from 0 to 24 hours. Control cultures contained the same diluent dilution as the test compounds. Completed circle (*).

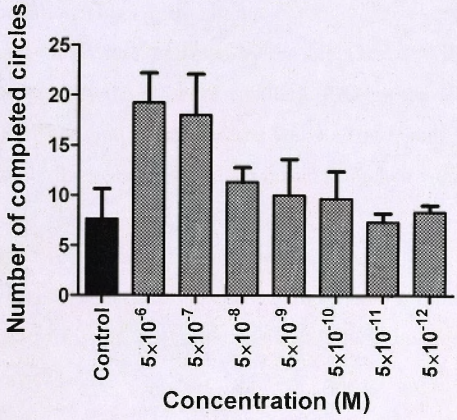


Figure 4.8. Effect of synthetic *FK1* on HMEC tube formation assay on Matrigel.

Effect of synthetic ***FK1***, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on HMEC tube formation was measured as number of completed circles after 6 hours culture. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=4). *, $P \leq 0.05$.

4.4.4. Effect of synthetic *FK1* on endothelial cell migration (wound healing assay)

An endothelial cell migration assay based on cell migration into a denuded area was employed to determine the effect of synthetic ***FK1*** on endothelial cell migration. Cell migration is a critical event in angiogenesis and wound healing processes and these tests were designed to measure the effects of ***FK1*** on *in vitro* cell migration.

In this assay, a wound was created in a confluent monolayer of human microvascular endothelial cells (HMEC) (section 2.2.11). Subsequently, the extent and speed of wound closure was monitored microscopically every 2 hours. HMEC migration into the cleared space was evident after 4 hours of culture and the wound fully recovered within 18-20 hours of incubation (Fig. 4.9). Images were taken every 2 hours using the IncuCyte machine and these were analysed by the IncuCyte and Prism software, using an unpaired t-test, to quantify the effect of synthetic ***FK1*** on the width of wounds from 0 to 26 hours of culture for control and treated wells. The results were then expressed as percentage wound confluence relative to the initial scratched wound mark.

Synthetic ***FK1*** appeared to have no measurable effect on the speed and extent of wound closure (Fig. 4.10). This experiment was repeated six times with six replicates each time and synthetic ***FK1*** was found to consistently have no significant effect on the wound healing response.

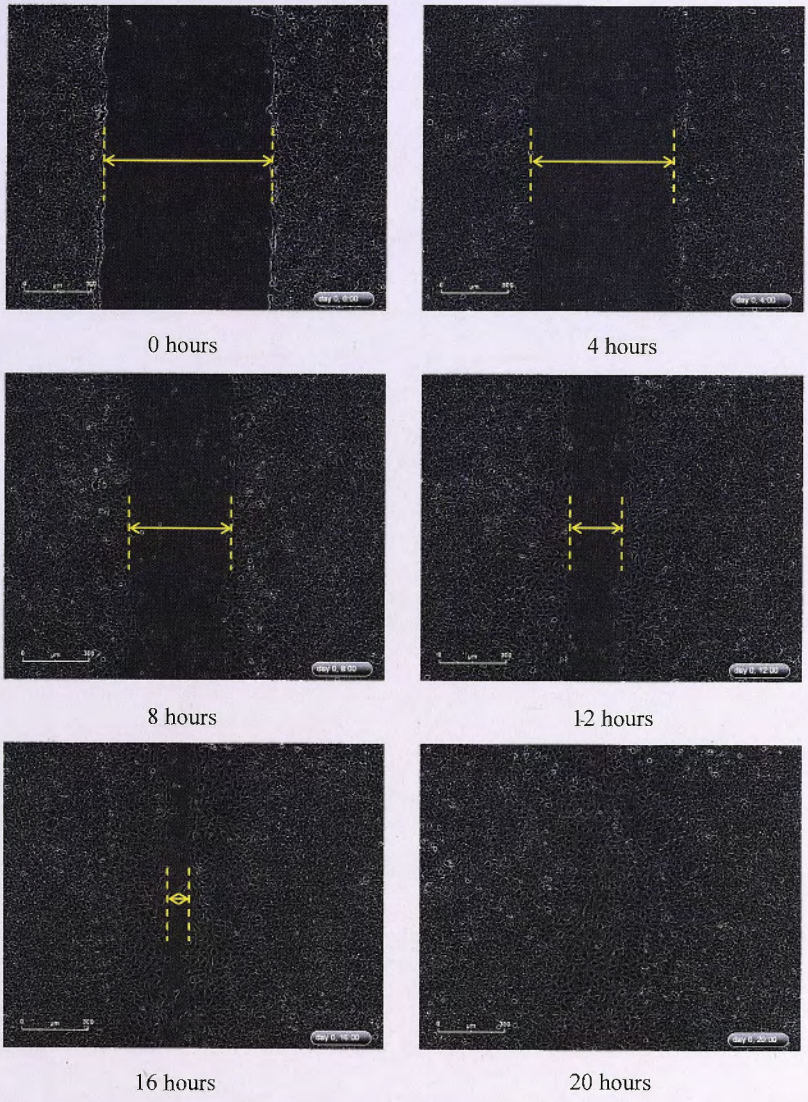


Figure 4.9. Time course of HMEC wound healing assay.

Wounds were made in HMEC monolayer in each well of a 96-well plate using the IncuCyte wound maker. Cell migration towards the denuded area was recorded every 2 hours and wound recovery was complete within 18-20 hours.

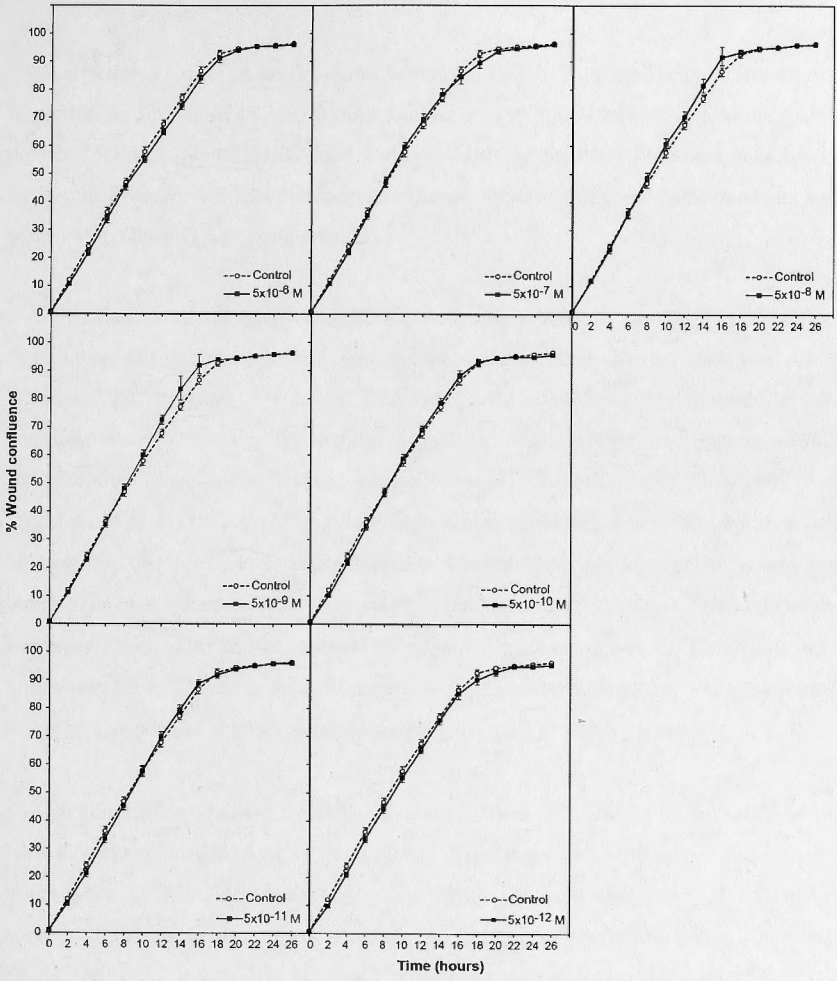


Figure 4.10. Effect of synthetic *FK1* on HMEC wound healing assay.

Effect of synthetic ***FK1***, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on wound recovery was measured as % wound confluence from 0 to 26 hours relative to the initial wound mark. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=6). No significant difference in cell adhesion was observed.

4.4.5. Effect of synthetic *FK1* on endothelial cell adhesion (Rose Bengal assay)

As discussed earlier in section 4.4.4, synthetic ***FK1*** had no significant effect on cell migration as measured by the wound healing assay, but it was able to significantly enhance HUVEC and HMEC tube formation on the artificial basement membrane, Matrigel. Therefore, it was pertinent to examine the effect of these compounds on cell adhesion to chosen ECM components.

Cell adhesion of the endothelial human cell line, HMEC to the ECM components, fibronectin and vitronectin, was determined using a Rose Bengal adhesion assay (section 2.2.13). First, fibronectin and vitronectin concentrations required for cell adhesion were optimised by coating the plastic wells of the microplates with a concentration range. The coating concentration of fibronectin and vitronectin was varied from 0 to 10 μg/mL to establish the concentrations where sub-optimal cell binding was observed. At such conditions it was reasoned that both enhancement and inhibition of cell adhesion may be detected in the presence of synthetic ***FK1*** at different concentrations. The results showed the optimal concentrations of fibronectin and vitronectin for cell binding were 10 μg/mL and 5 μg/mL, respectively, and sub-optimal cell binding occurred at concentrations ranging from 0.313 to 2.5 μg/mL (Fig. 4.11).

The effect of synthetic ***FK1*** at concentrations from 5×10^{-6} M to 5×10^{-12} M on HMEC endothelial adhesion to immobilised fibronectin and vitronectin was initially tested after 60 minutes incubation. The results showed that none of the tested concentrations had a significant effect on the binding of HMEC to either 10 μg/mL fibronectin (Fig. 4.12B) or to 5 and 10 μg/mL vitronectin (Fig. 4.13A and B). Moreover, similar results were obtained when the incubation time was decreased to 30 minute incubation for fibronectin (Fig. 4.12A).

In order to determine the cell adhesion rate, similar cell adhesion experiments were performed using a time course of incubations from 0-25 minutes. The results demonstrated a very fast cell binding response for fibronectin (Fig. 4.14) and gradual increase in adhesion to vitronectin, starting from 7.5 minutes (Fig 4.15). Furthermore, no significant effect of ***FK1*** on the rate of HMEC cell adhesion to fibronectin or vitronectin was observed for the tested concentrations at the different time points.

Overall the results demonstrated that the pro-angiogenic synthetic ***FK1*** had no effect on the adhesion of HMEC endothelial cell line to fibronectin or vitronectin at the time points tested, i.e., from 2.5 to 60 minutes incubation.

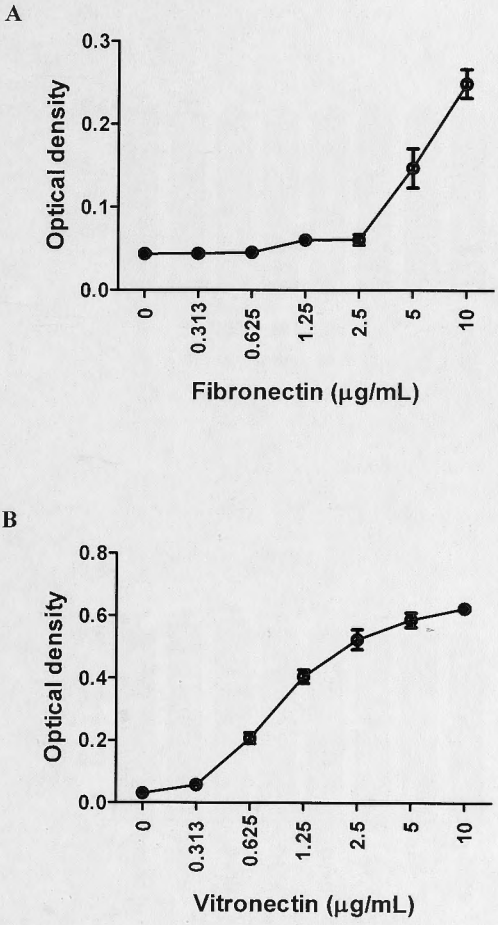


Figure 4.11. Optimisation of fibronectin and vitronectin concentrations used to coat wells for the Rose Bengal adhesion assay using HMEC.

HMEC adhesion to fibronectin (A) and vitronectin (B) was measured as optical density of Rose Bengal staining of adherent cells. Error bars represent SEM (n=6). The background binding in the absence of fibronectin and vitronectin was < 0.04 optical density unit.

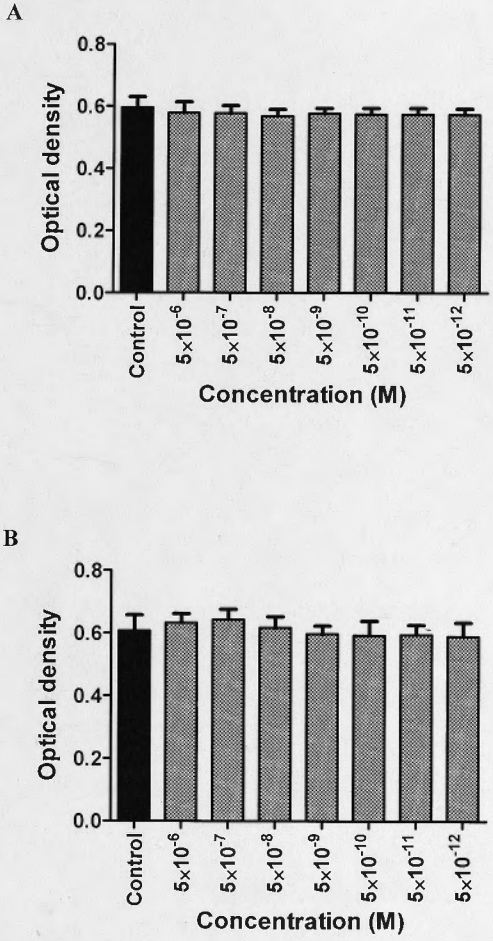


Figure 4.12. Effect of synthetic *FK1* on fibronectin-mediated HMEC cell adhesion.

Effect of synthetic ***FK1***, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on cell adhesion was measured after 30 minute (A) and 60 minute (B) incubation as optical density of Rose Bengal staining of adherent cells. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=4). No significant difference in cell adhesion was observed.

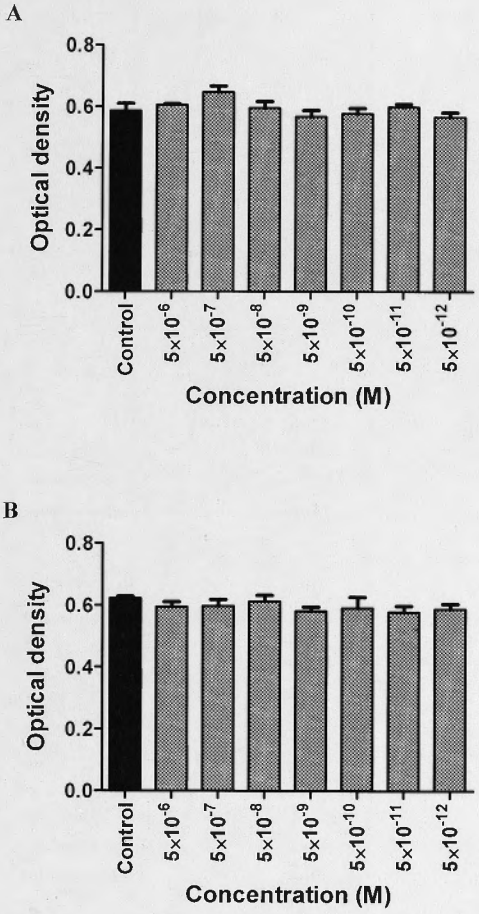


Figure 4.13. Effect of synthetic *FK1* on vitronectin-mediated HMEC cell adhesion.

Effect of synthetic ***FK1***, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on cell adhesion was measured using wells coated with 5 μg/mL (A) and 10 μg/mL (B) of vitronectin and after 60 minutes incubation as optical density of Rose Bengal staining of adherent cells. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=4). No significant difference in cell adhesion was observed.

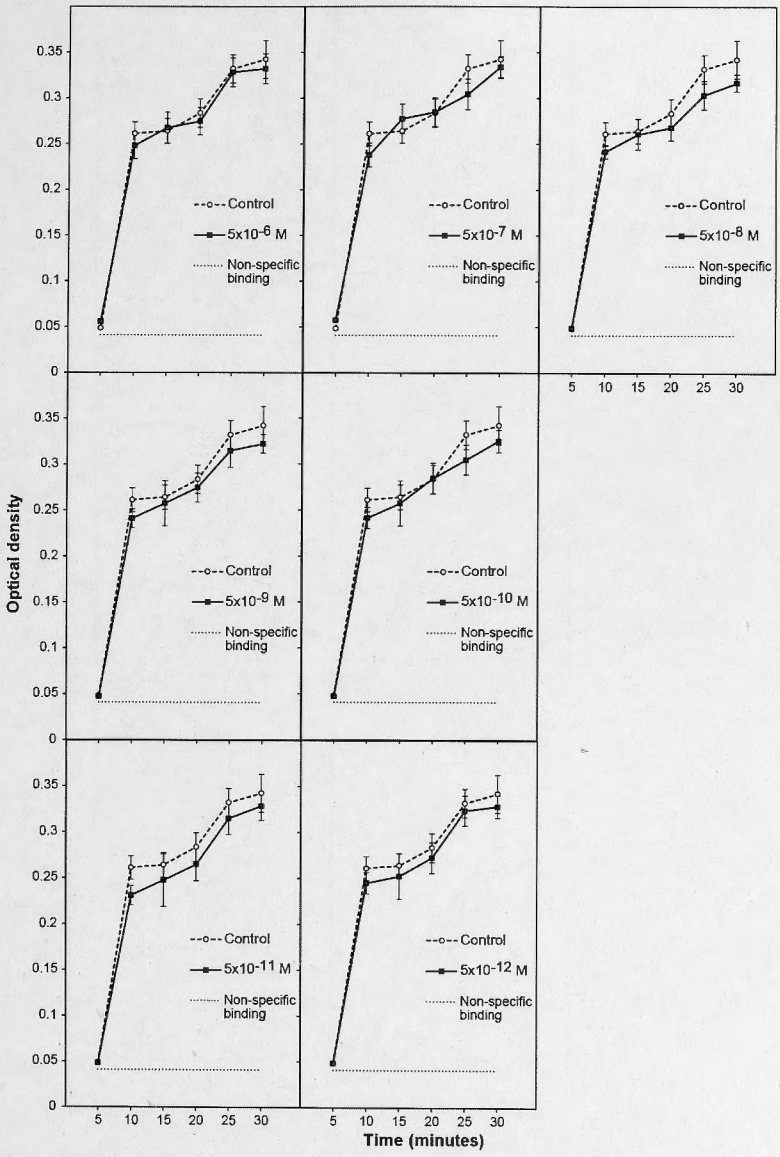


Figure 4.14. Time course of effect of synthetic *FK1* on fibronectin-mediated HMEC cell adhesion.

Effect of synthetic ***FK1***, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on cell adhesion was measured using wells coated with 10 µg/mL of fibronection and after 5-30 minutes incubation as optical density of Rose Bengal staining of adherent cells. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=6). No significant difference in cell adhesion was observed.

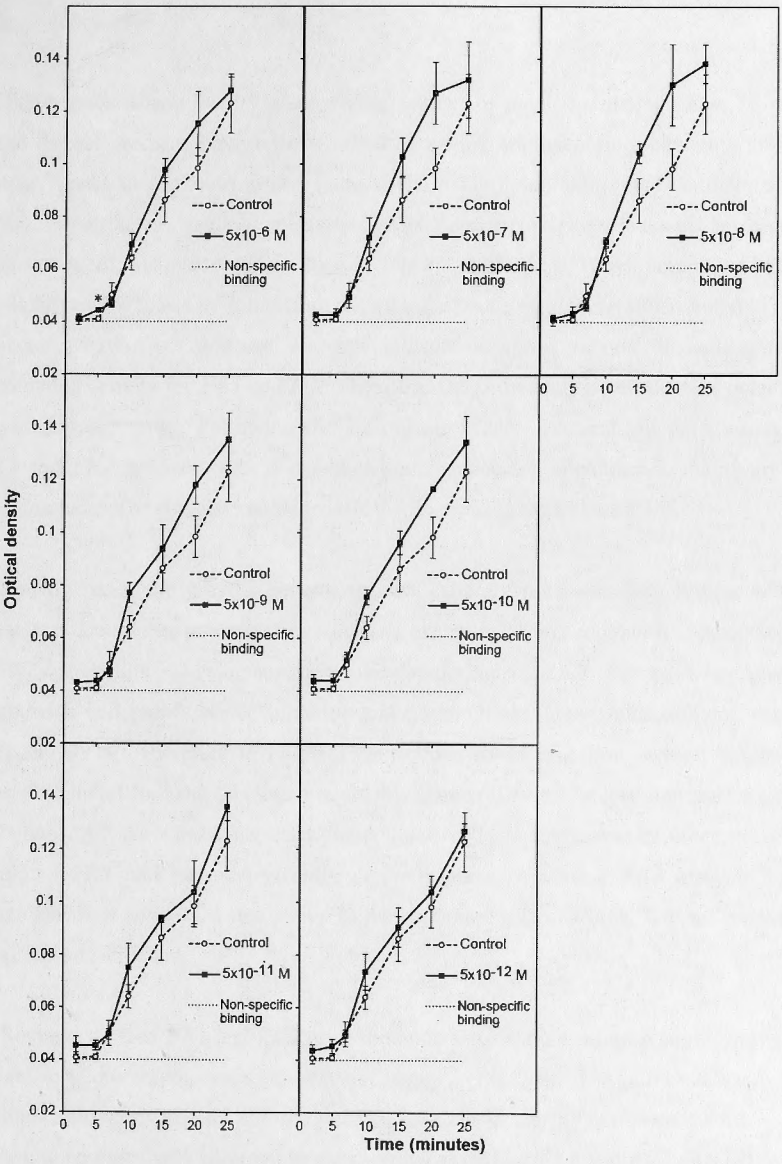


Figure 4.15. Time course of effect of synthetic *FK1* on vitronectin-mediated HMEC cell adhesion.

Effect of synthetic ***FK1***, at concentrations from 5×10^{-6} M to 5×10^{-12} M on cell adhesion, was measured using wells coated with 5 µg/mL vitronectin and after 2.5-25 minutes incubation as optical density of Rose Bengal staining of adherent cells. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=4). No significant difference in cell adhesion was observed

4.5. Discussion

Plants have a long history of providing sources of medicinal therapeutics. To date some natural products have been reported as having angiogenesis modulating effects (Sagar, Yance et al. 2006). These include vincristine, vinorelbine and taxol (Pezzuto 1997). Nevertheless, finding new classes of pro- and anti-angiogenic agents has been a major area of pharmacological interest (Newman 2011). These compounds may modulate angiogenesis by interacting with the multitude of pathways that underpin this process. Extensive literature searches indicate no prior reports of angiogenesis modulating activity for ***FK1*** or ***FK2***. Therefore, they constitute a new class of potential pro-angiogenic drugs. Following the identification and structural characterisation of ***FK1*** and ***FK2*** from soybean as pro-angiogenic molecules in chapter 3, the ability of these molecules to modulate angiogenesis was explored in this chapter.

Angiogenesis, the process generating new capillary networks from mature blood vessels, is a complex process of overlapping events including activation of endothelial cells, degradation of the basement membrane surrounding the existing vessel, endothelial cell proliferation, migration and differentiation into a mature blood vessel (Pepper 1997). Therefore to pinpoint the cellular mode of action, several bioassays needed to be utilised and this was done in this chapter. Due to the low concentrations of ***FK1*** and ***FK2*** in soybean materials, the cellular mode of action was explored utilising synthetic ***FK1*** and an independently derived source of natural ***FK2*** isolated from *Bretschneidera sinensis*, a tree native to the south and east of China, Taiwan, northern Thailand and Vietnam.

Soybean derived ***FK1*** and ***FK2*** were shown to significantly enhance angiogenesis in the rat aorta pro-angiogenesis bioassay in Chapter 3 (Fig. 3.8). The activity of synthetic commercially-derived ***FK1*** and the purified naturally-occurring compound, ***FK2,*** were shown to be essentially identical to the naturally occurring ***FK1*** and ***FK2*** purified from soybean using the RARM. The pro-angiogenic activity of synthetic ***FK1*** and independently isolated ***FK2*** were confirmed at concentrations ranging from 5×10^{-6} M to 5×10^{-9} M (Fig. 4.2). Interestingly, the activity of both compounds increased with increasing dilution with both peaking at 5×10^{-8} M and activity then tapering off with increasing dilution. The decrease in activity at concentrations higher than 5×10^{-8} M suggests that further increases in concentration may not have had significant effects on

angiogenesis. Indeed it is possible that at higher concentrations both that ***FK1*** and ***FK2*** may have opposite effects, or possibly toxic effects on endothelial cells, so it is important to define the therapeutic range of these molecules.

Although *in vitro* angiogenesis assays, such as the RARM, can be carried out rapidly and can provide initial valuable information about the overall biological activity, multiple biological assays need to be employed to obtain a comprehensive understanding of the activity of test compounds (Auerbach, Lewis et al. 2003). In order to investigate the cellular mode of action of synthetic ***FK1*** and ***FK2***, several *in vitro* assays portraying major steps in angiogenesis, including endothelial cell proliferation, migration, tube formation and adhesion to ECM components have been employed in sections 4.4.1 to 4.4.5. These steps can be studied *in vitro* while representing possible target interactions for these angiogenesis modulating molecules. In addition, *in vitro* studies on endothelial cells have been considered as good models for angiogenesis studies and for predicting possible *in vivo* effects of desirable vascular agents (Galbraith, Chaplin et al. 2001).

Initially, endothelial cell proliferation assays were performed using serum-starved HUVEC in the presence and absence of bFGF (12.5 ng/mL). The idea behind using bFGF, a potent mitogenic and pro-angiogenic growth factor, was to explore whether ***FK1*** and ***FK2*** can modify the mitogenic activity of bFGF. Previously, in the Nod factors studies, bFGF was used at a concentration of 25 ng/mL (Susanti 2011; Djordjevic, Bezos et al. 2013). However, the pro-angiogenic activity of ***FK1*** and ***FK2*** was considerably higher than the Nod factor derivatives (Susanti 2011), and thus the concentration of bFGF was lowered to 12.5 ng/mL in the studies described in this chapter. Using sub-optimal concentrations of bFGF in the endothelial cell proliferation assays allowed either enhancing or inhibiting effects of the test compounds to be measured. Generally, ***FK2*** was significantly more active than ***FK1*** in the HUVEC proliferation assay. ***FK2*** only significantly enhanced HUVEC proliferation in the absence of bFGF and the apparent raised levels of proliferation recorded with ***FK1*** were not significant. Also, in the presence of bFGF, synthetic ***FK1*** showed significant enhancement of HUVEC proliferation at concentrations ranging from 5×10^{-6} M to 5×10^{-8} M, whereas ***FK2*** significantly enhanced proliferation at all concentrations tested (5×10^{-6} M to 5×10^{-12} M) (Fig. 4.3). This suggests that both ***FK1*** and ***FK2*** can significantly enhance bFGF-induced cell proliferation, with ***FK2*** also possibly

activating proliferation in a bFGF-independent manner. Moreover, ***FK1*** significantly enhanced HUVEC and HMEC tube formation on an artificial ECM (Matrigel) (Fig. 4.6 – 4.7). However synthetic ***FK1*** showed no activity in the cell migration (wound healing) assay (Fig. 4.10) or also cell adhesion to the ECM components, fibronectin and vitronectin (Fig. 4.11 – 4.15).

Activation of FGFRs by bFGF (FGF-2) induces endothelial cell proliferation, migration and tube formation and also changes the expression and/or activation of integrins (Fig. 4.16) (Boilly, Vercoutter-Edouart et al. 2000; Turner and Grose 2010). Therefore, as synthetic ***FK1*** did enhance HUVEC proliferation and tube formation in the absence of bFGF, and as the HUVEC proliferation was even greater in the presence of bFGF, it can be hypothesised that these molecules have similar functionsto bFGF in enhancing angiogenesis directly rather than through activation of integrin-mediated attachment.

Activation of the FGF receptors, FGFR-1 or FGFR-2, by angiogenic FGFs including bFGF (FGF-2) typically results in endothelial cell proliferation. The FGFR-bFGF interaction leads to activation of several parallel signalling pathways after phosphorylation of the receptor following recruitment of Shc, FRS2, and Crk adaptor molecules (Cross and Claesson-Welsh 2001). Moreover, in addition to activation of the MAPK signalling pathway, activation of protein kinase C (PKC) is also required for a full mitogenic response of bFGF in endothelial cells (Presta, Tiberio et al. 1991) as down-regulation of PKC abrogates bFGF-induced endothelial cell proliferation. However, this does not affect urokinase-type plasminogen activator (uPA) up-regulation as it has been reported that phosphorylation of distinct tyrosine residues in FGFR-1 mediates the mitogenic and uPA inducing activity of bFGF (Dell'Era, Mohammadi et al. 1999). This is expected as uPA is one of the key molecules involved in ECM degradation during cell migration (Boilly, Vercoutter-Edouart et al. 2000). Therefore, the findings above suggest that the MAPK and PKC biological responses can be dissociated at the molecular level (Presta, Dell'Era et al. 2005; Turner and Grose 2010). It is possible that ***FK1*** affects either MAPK or PKC pathways or their downstream signalling pathways but as it significantly enhanced HUVEC proliferation mostly in the presence of bFGF, it can be hypothesised that it might also potentiate bFGF activity.

Integrins are key cell adhesion molecules involved in cell migration and differentiation in angiogenesis along with cadherins and ephrins. They are heterodimers with non-covalent dimerisation of α and β subunits that can form more than 24 combinations to ligate one or several ECM proteins (Table 4.1) (Liekens, De Clercq et al. 2001; Otrock, Mahfouz et al. 2007; Barczyk, Carracedo et al. 2010).

Once the specific matrix protein becomes available in the microenvironment of endothelial cells, and integrin ligation occurs, integrin-mediated signalling typically develops as a function of crosstalk between integrins and specifically activated cytokine or growth factor receptors. These signalling pathways result in cell survival, migration and differentiation (Weis and Cheresh 2011). For instance, αV and β3 are only expressed by angiogenic endothelial cells forming αVβ3, a receptor for both fibronectin and vitronectin and αVβ5 which binds to vitronectin (Feldinghabermann, Mueller et al. 1992; Filardo, Brooks et al. 1995; Liekens, De Clercq et al. 2001; Serini, Valdembri et al. 2006). Blocking αVβ3 and αVβ5 integrins has been found to block growth factor and tumour-induced angiogenesis (Eliceiri and Cheresh 2001; Liekens, De Clercq et al. 2001). Also, integrin α5β1 which is the main receptor for fibronectin, has also been reported to regulate vascular development and tumour angiogenesis (Serini, Valdembri et al. 2006).

Interestingly bFGF has been found to regulate the expression of different integrins, including αVβ3 and cadherins in cell-ECM and cell-cell adhesions (Presta, Dell'Era et al. 2005). Moreover, increasing the affinity of integrins for their ligands is another mechanism that cells can use to alter their adhesive response. For example, αVβ3 affinity has been reported to be modulated during angiogenesis and also selectively blocking high affinity αVβ3 could interrupt EC migration (Hood and Cheresh 2002).

Integrins are also involved in regulating the activities of proteolytic enzymes that hydrolyse or degrade the basement membrane by activating specific matrix metalloproteinases (MMP). For example, MMP-2 and MMP-9, which have the highest activity against type IV collagen (main constitute of basement membrane), are elevated in angiogenic endothelial cells (Weis and Cheresh 2011). Interestingly, integrin αvβ3 has been shown to bind to MMP-2, thus localizing MMP-2 mediated matrix degradation to the EC surface (Liekens, De Clercq et al. 2001; Otrock, Mahfouz et al. 2007).

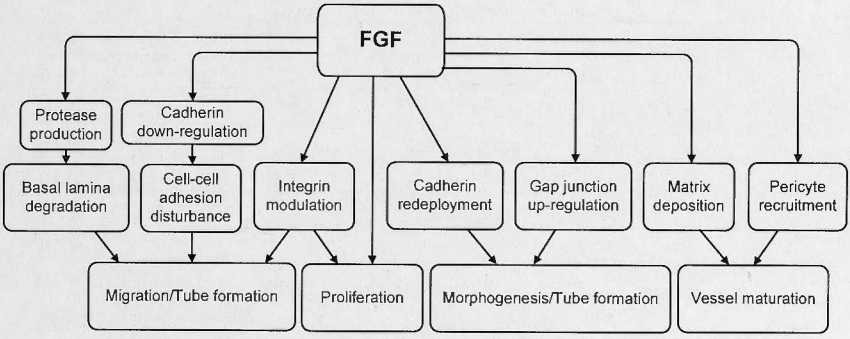


Figure 4.16. Schematic representation of events triggered by FGFs in endothelial cells in angiogenesis.

Adapted from (Presta, Dell'Era et al. 2005).

Table 4.1. Human integrins and their ECM ligands.

Integrins	ECM ligands
α1β1 (CD49a, VLA1)	Collagens (IV, I, IX)
α2β1 (CD49b, VLA2)	Collagens (IV, I, IX)
α3β1 (CD49c, VLA3)	Laminins
α4β1 (CD49d, VLA4)	Fibrinectin VCAM-1
α5β1 (CD49e, VLA5)	Fibronectin
α6β1 (CD49f, VLA6)	Laminins
α7β1	Laminins
α8β1	Fibronectin, vitronectin, nephronectin
α9β1	Tenascin-C, VEGF-C, VEGF-D
α10β1	Collagens (IV, VI, II, IX)
α11β1	Collagens (I, IV, IX)
αLβ2 (CD11a)	ICAM-1, -2, -3, -5
αMβ2 (CD11b)	iC3b, fibrinogen+more
αXβ2 (CD11c)	iC3b, fibrinogen+more
αDβ2 (CD11d)	ICAM-3, VCAM-1
αIIBβ3 (CD41, GpIIb)	Fibrinogen, fibronectin
α6β4	Laminins
αVβ1 (CD51)	Fibronectin, vitronectin
αVβ3	Fibronectin, vitronectin, fibrinogen
αVβ5	Vitronectin
αVβ6	Fibronectin, TGF-β-LAP
αVβ8	Vitronectin, TGF-β-LAP
αEβ7 (CD103, HML-1)	E-cadherin
α4β7	Fibronectin, VCAM-1, MadCAM-1

Adapted from (Barczyk, Carracedo et al. 2010).

Also, stimulation of endothelial cells by bFGF enhances formation of cell surface membrane vesicles containing MMP-2 and MMP-9 together with the two MMP inhibitors TIMP-1 and TIMP-2. These vesicles stimulate capillary-like structure formation when added to endothelial cells on Matrigel (Taraboletti, D'Ascenzo et al. 2002). Therefore, endothelial cell-ECM adhesion experiments using fibronectin and vitronectin were conducted to explore whether ***FK1*** could affect the adhesion process via these two ligands.

The results from HMEC-ECM adhesion in the presence of ***FK1*** showed no significant enhancement of endothelial cell-fibronectin/vitronectin binding and suggested that the ***FK1*** pro-angiogenic response is not an integrin-fibronectin/vitronectin mediated process. These results can also explain the results obtained from the migration assay, where ***FK1*** did not enhance the motility of endothelial cells.

In contrast, the synthetic ***FK1*** significantly enhanced endothelial tube formation on Matrigel. Matrigel is gelatinous mixture of ECM proteins, supplemented with laminin, collagen type IV, HSPG and various growth factors (Conn 2012). Integrin α6β4 has been reported to be an angiogenesis-specific integrin which binds to laminin as well as α6β1 (Serini, Trusolino et al. 1996; Hood and Cheresh 2002). Also, it has been shown that high levels of the α6 subunit are essential for the bFGF-induced HUVEC tubular morphogenesis (Bauer, Margolis et al. 1992; Matou, Helley et al. 2002). Moreover, heparan sulphates protect bFGF from proteolysis and degradation, and active bFGF will be released once proteoglycan degradation occurs (Rusnati and Presta 1996; Turner and Grose 2010). HSPG also promotes bFGF binding to FGFR and its subsequent activation (Powers, McLeskey et al. 2000; Schlessinger, Plotnikov et al. 2000).

Furthermore, it has been reported that integrin activation by their ligands can lead to stimulation of a wide range of intracellular signalling pathways including activation of Ras, MAPK, FAK, Src, Rac/Rho/cdc42 GTPase, PKC and PI3K. As mentioned earlier, many of these signalling events are also activated following growth factor stimulation (FGF-FGFR interaction). These overlapping signalling phenotypes indicate the coordination of endothelial cell adhesion with engagement of the growth factor receptors to regulate endothelial cell responses during angiogenesis (Eliceiri and Cheresh 2001). Thus, it has been suggested that angiogenic factors, either stimulators

or inhibitors, can modulate integrin function (Serini, Valdembri et al. 2006). Therefore, based on the above findings, it can be suggested that ***FK1*** might be modulating α6β1 integrin expression or enhancing its interaction with bFGF and bFGF receptor and/or it might potentiate the activity of low concentrations of bFGF present in Matrigel or HIFCS. Also, during tube formation both cell-ECM and cell-cell interactions (cadherins) are involved, therefore, it may be a possibility that ***FK1*** enhances endothelial cell-endothelial cell adhesion as well.

It should be mentioned that the adhesion and migration assays performed in this thesis used cell lines in which the integrins were already in an active, high affinity state so it is unlikely that pro-angiogenic ***FK1*** induces the integrin activation. This also may explain why ***FK1*** did not enhance cell adhesion to fibronectin and vitronectin as even if ***FK1*** could induce the high affinity integrin conformation, the adhesion assay would be unable to detect this as the integrins present on the cultured cells are already in the active, high affinity state. However, integrin-dependent cell adhesion might not be dependent on ligand-binding affinity. Bazzoni and Helmer (1998) showed that the regulation of α4β1 function by chemokines can be regulated by the actin cytoskeleton configuration (Bazzoni and Hemler 1998) suggesting that pro-angiogenic ***FK1*** might regulate integrin-mediated cell adhesion events in endothelial cell tube formation without altering the ligand-binding affinity.

In this study, it was demonstrated that ***FK1*** and ***FK2*** have pro-angiogenic activity. ***FK1*** and ***FK2*** are precursors for lignin which is a polymer found in the wood and the secondary cell walls of vascular plants (Novaes, Kirst et al. 2010; Vanholme, Demedts et al. 2010). Lignin is comprised of the monolignol units (ρ-coumaryl, coniferyl and synapyl alcohols) (Fig. 3.12) binding as dimers at C8 (C8-C8′) (lignans) and four other sites C5-C5′, C8-C5′, C4-*O*-C5′ and C8-*O*-C4′ (neolignans) (Bunzel, Ralph et al. 2001; Peterson, Dwyer et al. 2010) (Fig. 3.13). The *erythro*- and *threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohols are neolignans (Mander and Liu 2010) and with the exception of maize, were identified in all investigated cereal fibres (barley, millet, oat, rice, rye, spelt, wheat and wild rice) (Begum, Nicolle et al. 2004; Bunzel, Ralph et al. 2004).

Lignan (C8-C8′) and neolignan (C8-C5′) dimers have been reported to have a wide range of bioactivity in humans (Table 4.2) (Milder, Feskens et al. 2005; Van Miert, Van Dyck et al. 2005; Peterson, Dwyer et al. 2010). Lignans with C8-C8′ linkages occur

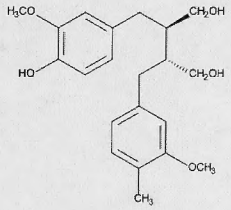
commonly in food (Fig. 4.17) (Umezawa 2007) and have also been shown to be precursors of mammalian lignans after fermentation by human large intestine microflora. For example, the plant lignans matairesinol, secoisolariciresinol, pioresinoland and syringaresinol can be converted to mammalian lignans enterolactone and enterodiol (Fig. 4.18) (Heinonen, Nurmi et al. 2001).

In plants, lignin macromolecules have crucial structural roles including the thickening of xylem cells which, in turn, helps plants to stand upright and withstand wind damage. Lignin polymers also provide a hydrophobic surface that allows plants to transport water, nutrients and extractives. Lignin's physical and chemical properties also serve as a barrier against the invasion of pests and pathogens (Campbell and Sederoff 1996; Novaes, Kirst et al. 2010).

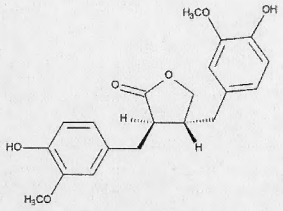
As mentioned above, lignins are present in a large variety of foods and are particularly abundant in cereal grains (Begum, Nicolle et al. 2004; Bunzel, Ralph et al. 2004) and nutritionally are components of insoluble dietary fibre (DeVries 2003). Dietary fibre is a macronutrient defined as the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine by microflora (Prosky 2000). It has been hypothesised that plant lignins can be a potential precursor of the mammalian lignans as a result of bacterial fermentation in the large intestine (Birt, Markin et al. 1998). However, this was not supported by the results of Birt and colleagues in 1998. They showed that rats fed with 5% purified wood lignin were unable to produce the mammalian lignans. They also showed no influence of lignin feeding on mammary cancer induced by N-methyl-N-nitrosourea (Birt, Markin et al. 1998). In contrast, Begum et al. (2004) showed that the feeding of synthetic lignin made from coniferyl alcohol (dehydropolymerase, DHP) to rats led to the detection of the mammalian lignan, enterolactone, in urine. They concluded that this resulted from the metabolism of lignin by the intestine microflora (Begum, Nicolle et al. 2004).

Table 4.2. Examples of biological activities of lignans in humans.

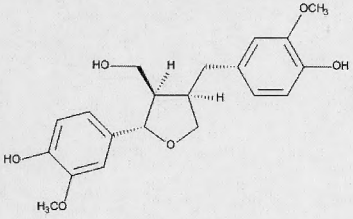
Biological activity	References
Anti-radical and anti-oxidant	(Suja, Jayalekshmy et al. 2004; Eklund, Langvik et al. 2005; Gulcin, Elias et al. 2006; Kancheva, Saso et al. 2012)
Postmenopausal breast cancer prevention	(Buck, Zaineddin et al. 2011; McCann, Hootman et al. 2012)
Anti-colon cancer	(Jenab and Thompson 1996; Hausott, Greger et al. 2003; Qu, Madl et al. 2005)
Anti- prostate cancer	(McCann, Gill et al. 2005; Landberg, Andersson et al. 2010)
Cardiovascular health	(Bloedon and Szapary 2004; Bloedon, Balikai et al. 2008; Prasad 2009; Peterson, Dwyer et al. 2010)
Cytotoxicity and anti-tumour effect	(Takasaki, Konoshima et al. 2000; Qu, Madl et al. 2005; Van Miert, Van Dyck et al. 2005; Zhou, Liu et al. 2009)
Anti-ovarian cancer	(McCann, Freudenheim et al. 2003)
Oestrogenic and anti-oestrogenic activities	(Mazur and Adlercreutz 2000; Aehle, Muller et al. 2011)
Anti-inflammation	(Hallund, Tetens et al. 2008; Baumgartner, Sosa et al. 2011)
Reducing blood pressure	(Cornish, Chilibeck et al. 2009; Peterson, Dwyer et al. 2010)
Cholesterol lowering	(Zhang, Wang et al. 2008; Fukumitsu, Aida et al. 2010)



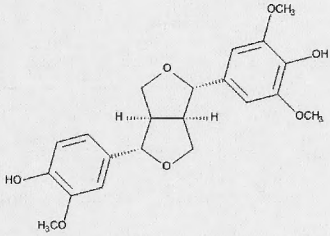
Secoisolariciresinol
Cashews, chickpeas, coffee, cranberry, flax, peas, sunflower seeds, wine



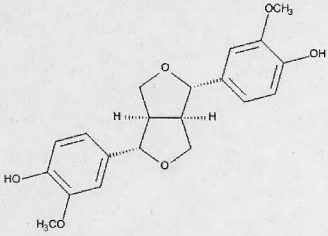
Matairesinol
Flax, oats, pineapples, rye, wine



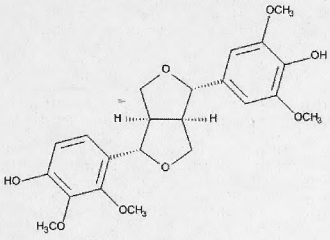
Lariciresinol
Buckwheat, eggplant, oats, pineapple, rye



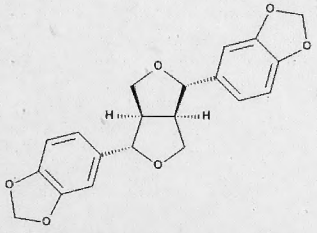
Medioresinol
Lemon, rye, sesame seeds



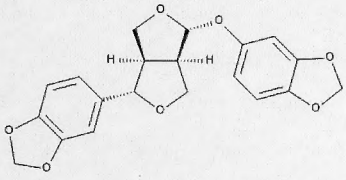
Pinoresinol
Asparagus, flax, lemon, rye



Syringaresinol
Asparagus, barley, buckwheat, millet, oranges, rye, wheat



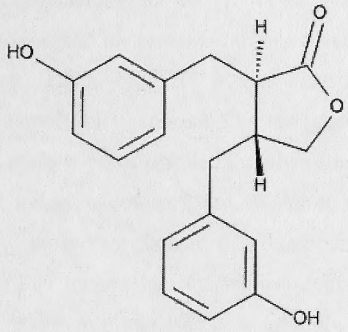
Sesamin
Sesame seeds



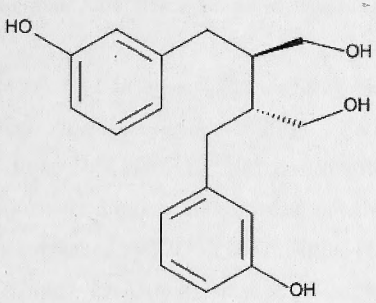
Sesamolin
Sesame seed lignan precursor

Figure 4.17. Structures and sources of some common dietary plant lignans.

Adapted from (Peterson, Dwyer et al. 2010).



Enterolactone



Enterodiol

Figure 4.18. Structures of mammalian lignans enterolactone and enterodiol.

There are only a few reports on bioactivity of *erythro*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK1***) and *threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK2***). A mixture of these two molecules was cytotoxic for human colon carcinoma (HCT-116) and human hepatocellular carcinoma (HepG2) cell lines at 30.2 and 57.3 μg/mL (80 and 152 μM), respectively (Lee, Song et al. 2007). These molecules were also shown to have a chemopreventative effect on prostate cancer by inhibition of prostate specific antigen (PSA) secretion (IC50, FK1=109.3 and FK2=130.5 μM) and they also suppressed androgen receptor (AR) expression by the prostate cancer cell line LNCaP cells at 100 μM. In this study it was suggested that they suppress the androgen receptor signalling pathway and induce apoptosis (Han, Wang et al. 2008). It is difficult to compare these studies to those described in this thesis since the concentrations used were a minimum of 16 fold higher than the highest concentrations described in this chapter. However, the results from the ***FK1*** and specially ***FK2*** rat aorta angiogenesis and HUVEC proliferation assays showed that decreasing the concentration from 5×10^{-6} M to 5×10^{-8} M resulted in higher activity. This might suggest that increasing tested concentrations to the high μM range might reverse the pro-angiogenic response to an anti-angiogenic effect. However, concentrations higher than 1 μM are very unlikely to occur in plant-derived natural products or to be detected in human plasma and tissues after lignin digestion (Hollman 2004; Perez-Vizcaino, Duarte et al. 2012).

We also tested synthetic ***FK1*** in an anti-malaria drug bioassay to investigate its possible inhibitory effect on *Plasmodium* viability. ***FK1*** showed no effect at concentrations ranging from 10^{-4} M to 10^{-14} M on parasite viability compared to artemisinin (known anti-malaria drug) as control (data not shown). These results are comparable with a study conducted in 2005 in which HPLC plant (*Grewia bilamellata*) fractions containing ***FK1*** and ***FK2*** (mix) showed no antimalarial activity against *P.faciparum* (Ma, Zhang et al. 2006).

In conclusion, it has been demonstrated that two lignin precursors, *erythro*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK1***) and *threo*-guaiacylglycerol-8-*O*-4′-coniferyl alcohol (***FK2***), possess pro-angiogenic activity in an animal model. The cellular mode of action of these molecules was then investigated by studying their effect on endothelial cell proliferation, migration, tube formation and adhesion to ECM components. It was found that the compounds enhanced endothelial cell proliferation and endothelial cell tube formation on an artificial ECM. These activities suggest that

these molecules have bFGF like stimulatory activity and may enhance angiogenesis via FGF signalling pathways.

Chapter 5

Identification of Pro-angiogenic Flavonoids, Their Structural Activity Relationship and Cellular Mode of Action

5.1. Introduction

Flavonoids are polyphenolic plant substances commonly found in the human diet especially in fruits, vegetables and beverages such as tea and red wine (Table 1.1) (Aherne and O'Brien 2002). Flavonoids are implicated in positively influencing a number of human conditions and disease by their anti-tumour, anti-inflammatory, anti-microbial and anti-oxidant activities (Ren, Qiao et al. 2003). However, the bioactivities of these compounds have been mostly studied at high concentrations (> 1 μM) in both *in vitro* and *in vivo* experiments and these concentrations have not been reported to be physiologically relevant. Therefore, reports on flavonoid structure activity relationship using high flavonoid concentrations (Middleton, Kandaswami et al. 2000; Havsteen 2002; Monasterio, Urdaci et al. 2004; Touil, Fellous et al. 2009; Lam, Alex et al. 2012) have to be interpreted with this in mind.

In this chapter, the pro-angiogenic activity of flavonoids at physiological concentrations (sub μM to mid nM levels) has been investigated first using an *in vitro* rat aorta pro-angiogenesis assay. The structural features of flavonoids that mediate their effects on angiogenesis were also investigated. Moreover, two flavonoids, genistein and naringenin, which have been found in the literature to be anti-angiogenic at concentrations above 1 μM, were found to be pro-angiogenic at lower concentrations. The bioactivity of these and other flavonoids was examined across a wide concentration range (5×10^{-6} to 5×10^{-12} M) that included concentrations shown to be present in human plasma. The cellular mode of action of these flavonoids was then studied using *in vitro* endothelial assays including cell proliferation, migration, tube formation and adhesion to ECM components.

5.1.1. Metabolism and absorption of flavonoids

Flavonoid glycosides are the predominant dietary source of flavonoids (Walle, Browning et al. 2005), although flavonoids from teas (also called tea flavonoids) occur as aglycones (Table 1.1) (Lee, Lambert et al. 2004; Walle 2004). Flavonoid glycosides are hydrolysed by the acidic conditions in the stomach and the resulting aglycones are absorbed into the circulatory system mainly from the small (Passamonti, Terdoslavich et al. 2009; Crozier, Del Rio et al. 2010), but also from the large intestine (Erlund, Meririnne et al. 2001) (Fig. 5.1). However, flavonoid glycosides such as genistein-7-*O*-

glucoside or quercetin-4'-*O*-glucoside can be hydrolysed to genistein and quercetin, respectively, in the oral cavity and absorbed by the oral epithelial cells (Walle, Browning et al. 2005).

Generally, the transport of flavonoids across the alimentary membranes is facilitated by both ATP-dependent pumps and ATP-independent transporters (Passamonti, Terdoslavich et al. 2009). In the small intestine, flavonoid-*O*-β-D-glycosides that survive passage through the stomach may be hydrolysed by lactase-phlorizin hydrolase (LPH) in the brush border of the epithelial cells or by a cytosolic β-glycosidase (CBG) within the epithelial cell. In order for CBG-catalysed hydrolysis to occur, the polar glycosides must be transported into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose transporter SGLT1 and further hydrolysed intracellularly (Day, DuPont et al. 1998; Gee, DuPont et al. 2000). The aglycones released in the brush border may enter the epithelial cells by passive diffusion as a result of their increased hydrophobicity (Day, Canada et al. 2000). Thus, there are two possible routes by which the glycoside conjugates are hydrolysed and the resultant aglycones appear in the epithelial cells, namely "LPH/diffusion" and "transport/CBG" (Crozier, Del Rio et al. 2010). In addition, it has been hypothesised that any remaining flavonoid glycosides may be hydrolysed by the gastrointestinal microflora in the distal part of the small intestine or the large intestine (Fig. 5.1) (Erlund, Meririnne et al. 2001).

After the aglycones enter the cellular environment they may be conjugated with glucuronic acid, sulphate and/or methylated during first-pass metabolism in the intestinal wall or in the liver. The enzymes responsible for these metabolic processes are UDP-glucoronosyltransferase, sulfotransferases and methyltransferases. *In vitro* studies of the bioactivity of these conjugated metabolites indicated that they are less active or totally inactive compared to the aglycones. However, the β-glucuronidase enzyme found in the vascular walls is able to hydrolyse the glucuronide conjugates allowing uptake of the aglycone by different tissues (Perez-Vizcaino, Duarte et al. 2012).

5.1.2. Bioavailability of flavonoids

Bioavailability is defined as the proportion of unchanged administered substance that reaches the systematic circulation (Passamonti, Terdoslavich et al. 2009). Flavonoids are not synthesised in animal cells, so their presence in animal tissues is derived entirely from the ingestion of plants or their derived products (Middleton, Kandaswami et al. 2000; Mennen, Sapinho et al. 2008). However, the level of dietary flavonoids varies greatly between individuals and countries, with the lowest average intake (2.6 mg/day) reported in Finland and the highest intake (68.2 mg/day) in Japan (Nijveldt, van Nood et al. 2001).

The first barrier to the absorption of flavonoids is the intestinal epithelium and enterocytes. Compounds with a relative molecular mass of more than 500 and able to form hydrogen bonds, are generally unable to cross biological membranes by passive diffusion (Lipinski, Lombardo et al. 2001) and thus the ingested glycosylated flavonoids, the major flavonoid component, are poorly absorbed at the gastro-intestinal level. Those that are absorbed into the blood stream then face the additional barriers of the cell plasma membranes of peripheral tissues. This is confirmed by a number of studies, showing that flavonoid bioavailability is mostly <1% of the administered dose (Kris-Etherton, Lefevre et al. 2004). In the other words, the intestinal absorption of flavonoids can be high; however, the plasma concentration of any individual molecule rarely exceeds 1 μM after the consumption of 10-100 mg of a single compound (Scalbert and Williamson 2000).

Thus although the flavonoid aglycones have a well demonstrated *in vitro* biological activity as antioxidants, anti-inflammatory, anti-proliferative and anti-angiogenic agents these studies have been conducted at concentrations ranging from than 25 μM to 200 μM (Rice-Evans 2004; Comalada, Ballester et al. 2006). Therefore, these activities are unlikely to be relevant *in vivo* as the concentration of flavonoids in plasma and tissues following oral ingestion is in the nM to μM range (i.e., less than 1 μM for normal diet) (Hollman 2004; Williamson and Manach 2005; Crozier, Del Rio et al. 2010; Perez-Vizcaino, Duarte et al. 2012).

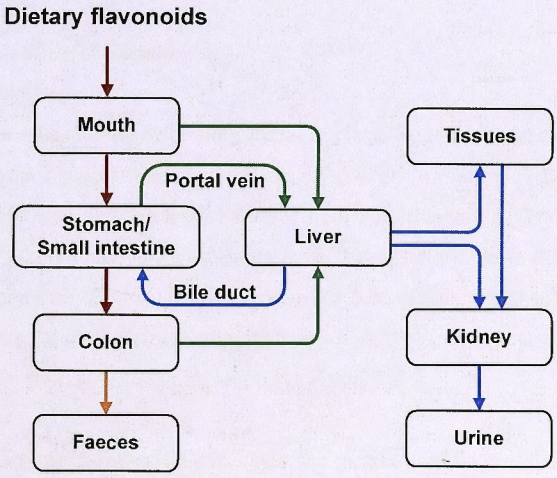


Figure 5.1. Possible routes for absorption and excretion of dietary flavonoids in humans.

Dietary flavonoids (glycosides) are mostly hydrolysed and then absorbed by the body from the small intestine as aglycones. Remaining glycosides will be carried to the colon where they may be hydrolysed by the endemic microflora. Absorbed flavonoids can be metabolised to glucuronides or sulphates and/or methylated in the epithelial cells of the gut and in the liver for circulation around the body or for excretion into the intestine via the bile duct. The circulating conjugates can be hydrolysed for uptake into various tissues or excreted via the kidneys. Metabolism of the flavonoid skeleton to water soluble phenolic acids can occur in the tissues (excreted via the kidney) or in the gut. Glycosides (→), aglycones (→), glucuronides/sulphates and aglycones (→), phenolic acids and undigested glycosides (→). Adapted from (Scalbert and Williamson 2000).

Thus, the concentrations of individual flavonoids and their biologically active conjugates may not be high enough after occasional intake to explain their health benefits. However, low concentrations may not necessarily indicate lack of biological activity for a highly potent substance. Moreover, accumulation may occur with regular dietary intakes (half-lives from 8-24 hours), which may result in sufficiently active flavonoid concentrations being achieved (Nijveldt, van Nood et al. 2001; Perez-Vizcaino, Duarte et al. 2012).

5.1.3. Bioavailability of naringenin

Naringenin is a flavanone, commonly found in citrus fruits and tomatoes and mainly occurs as glycosides such as naringenin-7-rhamnoglucoside (naringin) and naringenin-7-*O*-glucoside (Krause and Galensa 1992; Erlund, Meririnne et al. 2001; Bugianesi, Catasta et al. 2002; Yamamoto, Yoshimura et al. 2004). Naringin is the predominant flavanone in grapefruit (*Citrus paradisi*) (up to 10% of the dry weight/ 100-500 mg/l) and is responsible for the bitterness of grapefruit juices (Ortuno, Garciapuig et al. 1995; Manach, Scalbert et al. 2004; Jagetia, Jagetia et al. 2007).

Naringenin glycosides ingested with food are metabolised by the human intestinal microflora to the aglycone naringenin (Felgines, Texier et al. 2000; Matsumoto, Ikoma et al. 2004). Citrus flavonoids have been reported to be anti-inflammatory, anti-carcinogenic, cholesterol lowering and neuro-protective (Benavente-Garcia, Castillo et al. 1997; Montanari, Chen et al. 1998; Lee, Jeong et al. 2001; Heo, Kim et al. 2004; Galluzzo, Ascenzi et al. 2008; Mulvihill and Huff 2010). In addition, naringenin is a phyto-oestrogen and, like certain isoflavones, can bind to oestrogen receptors (Harris, Besselink et al. 2005) or inhibit aromatase activity, the rate limiting enzyme in the conversion of androgens to oestrogens (Edmunds, Holloway et al. 2005). Analysis of plasma and urine showed only the presence of the naringenin aglycone and the sulfoconjugated derivative (90%) and also glucuronides (Felgines, Texier et al. 2000; Erlund, Meririnne et al. 2001; Gardana, Guarnieri et al. 2007).

In a study conducted by Erlund et al. in 2001 showed that plasma naringenin concentration was measured after a baseline period (2 weeks), on a low vegetable diet (5 weeks, containing low amounts of vegetables and fruit, and no citrus) and a high vegetable diet (5 weeks, containing high amount of vegetables and fruit, including

moderate amounts of citrus) (Erlund, Meririnne et al. 2001). The daily baseline intake of naringenin was found to be 3 mg while the high vegetable diet provided 29 mg of naringenin and the low vegetable diet contained no flavanones. The plasma naringenin concentrations for the low and high vegetable diet were below the limit of detection and 1.129×10^{-7} M (112.9 nM), respectively. Later, these results were confirmed by Gardana et al. in 2007 and it was shown that the maximum naringenin concentration in plasma was 1.25×10^{-7} M (125 nM), 5 hours after taking 300 mL of orange juice and was undetectable in plasma after 24 hours(Gardana, Guarnieri et al. 2007). The plasma concentrations (ng/mL) were also comparable with those found in two other studies (Manach, Morand et al. 2003; Manach, Williamson et al. 2005).

Plasma and urine studies showed that naringenin can be detected within 2-4 hours of oral administration. However, after 5 hours there was found to be a second increase in plasma naringenin levels corresponding to naringenin released and absorbed from the glycoside (naringin; naringenin-7-rhamnoglucoside) in the large intestine (Felgines, Texier et al. 2000; Gardana, Guarnieri et al. 2007). These studies indicated that the flavanone did not accumulate in plasma, however, distribution and accumulation of flavanones in other tissues could not be ruled out, because compounds taken into cells could be released back into the bloodstream at concentrations below the limit of detection of the analytical method, or as metabolites or degradation products.

5.1.4. Bioavailability of genistein

The isoflavones, genistein, daidzein and glycitin (B-ring attached to C3 rather than C2 position as in other flavonoids) have a very limited distribution in the plant kingdom being found only in leguminous species (Kaufman, Duke et al. 1997) mostly as the polar, water soluble 6-*O*-β-glucosides, -acetylglucoside or -malonylglucoside conjugates (Coward, Smith et al. 1998; King and Bursill 1998; Setchell 2000; Rice-Evans 2004). Worldwide, soybean (*Glycine max*) is the most common dietary source of isoflavones. However, they also occur in low levels in black beans (*Phaseolus vulgaris*) and green peas (*pisum sativum*).

After ingestion, the isoflavone glucosides are hydrolysed by both intestinal mucosal and bacterial β-glucosidases releasing the aglycones which are then either absorbed directly or further metabolised by the intestinal microflora in the large intestine into the

isoflavonoid microbial metabolites, dihydrodaidzein, dihydrogenistein, dihydroglycitein, *O*-desmethylangolensin, equol, 6-hydroxy-*O*-desmethylangolensin, 5'-methoxy-*O*-desmethylangolensin and dihydro-6, 7, 4'-trihydroxyisoflavone (Day, DuPont et al. 1998; Setchell, Brown et al. 2002; Walsh 2006).

The isoflavonoids also act as phytoestrogens and have a wide range of hormonal and non-hormonal activities in animals or *in vitro* (Cassidy, Hanley et al. 2000) suggesting the potential human health benefits to be derived from a diet rich in these compounds. They have similar chemical structure to oestrogens due to the phenolic rings and particularly the 4'-hydroxyl and been found to bind to oestrogen receptors (Wang, Sathyamoorthy et al. 1996; Zava and Duwe 1997). Genistein has shown positive results in treating breast cancer (Peterson and Barnes 1991; Ju, Doerge et al. 2002; Du, Yang et al. 2012), modulating angiogenesis by inhibiting tyrosine kinases and VEGF expression, release and activation (Akiyama, Ishida et al. 1987; Fotsis, Pepper et al. 1993; Buchler, Reber et al. 2004; Qi, Weber et al. 2011; Yu, Zhu et al. 2012) and inhibiting cell proliferation (Fotsis, Pepper et al. 1995; Comalada, Ballester et al. 2006; Qi, Weber et al. 2011) and cell migration (Tham, Gardner et al. 1998). Using *in vitro* models, they have been tested at concentrations in the range of 25-100 μM. It has been observed that the plasma concentration of genistein after consumption of single or multiple soy containing meals rises slowly and reaches a maximum value of 1-5 μM after 5.5-7.5 hours with a half-life of 9-11 hours and returned to undetectable levels after 24-35 hours (Xu, Wang et al. 1994; King and Bursill 1998; Cassidy 2006).

Thus, the observed *in vitro* pharmacokinetics of genistein is important with respect to possible use of this compound in therapeutic applications. Based on the likely daily intake of isoflavones, absorption from the gut, distribution to peripheral tissues and excretion, it is unlikely that the blood isoflavone concentrations, even in high soy consumers, would exceed 1-5 μM. The mean concentration of circulating genistein found in Japanese men (high consumers) was 492.7 nM compared to 33.2 nM in men from the UK (low consumers) (Tham, Gardner et al. 1998; Morton, Arisaka et al. 2002). Therefore, studying the effects of genistein at likely physiological concentrations (< 1 μM) became the area of interest in this project.

5.1.5. Flavonoid properties in angiogenesis

Flavonoids are known to have anti-carcinogenic, anti-microbial, anti-inflammatory, anti-allergic, anti-oxidant and anti-viral activities. They also are thought to lower the risk of human cardiovascular disease (Hollman and Katan 1999; Middleton, Kandaswami et al. 2000; Nijveldt, van Nood et al. 2001; Comalada, Ballester et al. 2006; Lam, Alex et al. 2012). Many studies of flavonoids have shown a structure-activity relationship (SAR), suggesting that the position and number of the hydroxyl and methoxyl moieties on the flavonoid A and B rings, along with the oxidation state of carbon 4 (C4=O), the presence of a double bond in ring C (C2-C3) and of glycosylation, may be important factors in conferring activity (Table 5.1) (Fotsis, Pepper et al. 1997; Kawaii, Tomono et al. 1999; Casagrande and Darbon 2001; Manthey and Guthrie 2002; Rodriguez, Yanez et al. 2002; Bagli, Stefaniotou et al. 2004; Kanadaswami, Lee et al. 2005; Mayr, Windhorst et al. 2005).

Flavonoids are also known to influence the otherwise tightly regulated process of angiogenesis. Several critical events in the process of angiogenesis have been shown to be inhibited by flavonoids. These include the proliferation and migration of both endothelial cells and smooth muscle cells from the vascular wall. In addition, flavonoids are also known to inhibit the expression of both VEGF and MMP-2, which are important partners in the angiogenic process (Fotsis, Pepper et al. 1997; Paper 1998; Benavente-Garcia and Castillo 2008).

The presence of the C2-C3 double bond on the C ring, conjugated with the 4-oxo function, has been shown to be a critical structural feature for the anti-proliferative activity of flavonoids (Rodriguez, Yanez et al. 2002; Comalada, Ballester et al. 2006). Subsequent studies showed that the presence of three or more hydroxyls in any of the rings of the flavonoid skeleton also significantly increased the anti-proliferative activity observed in B16F10 melanoma cell cultures (Martinez, Yanez et al. 2003). However, in comparing luteolin (flavone) with quercetin (flavonol) (quercetin has an additional 3-hydroxy; see Table 1.1), in different cell lines, it seems the 3-hydroxylation does not confer a greater anti-proliferative activity (Casagrande and Darbon 2001; Manthey and Guthrie 2002; Rodriguez, Yanez et al. 2002).

Methylation of the hydroxyls does not reduce the anti-proliferative capacity and even appears to increase it, because the activity shown by 7-3′-dimethylhesperetin (2 methoxyls) was higher than that shown by its homologues, hesperetin (1 methoxyl) and eriodictyol (no methoxyls; see structures in Table 1.1), suggesting that the presence of a methoxyl group in position C4 may be related to greater cytostatic activity (Benavente-Garcia and Castillo 2008). Furthermore, the high anti-proliferative effects of tangeretin (4 methoxyls on the A ring and one on the C ring; Table 1.1) in many cancer cell lines (Hirano, Abe et al. 1995; Manthey and Guthrie 2002; Kanadaswami, Lee et al. 2005) shows being less polar and having a planar structure may play a critical role in the biological activity, enhancing flavonoid permeability through biological membranes and its binding properties (Manthey and Guthrie 2002).

The *in vivo* structure-activity relationship analysis indicated that a flavonoid with a methoxylated group at the C3′ (C3′-OCH3) position possesses a stronger anti-angiogenic activity, whereas the absence of a methoxylated group at the C8 position results in lower lethal toxicity in addition to enhancing the anti-angiogenic activity. Also, methylation of the OH groups at the C4′, C5, C6 and/or C7 positions may increase the anti-angiogenic activity (Lam, Alex et al. 2012).

Naringin (naringenin-7-O-glycoside) in comparison with naringenin displayed no detectable anti-angiogenic activity at concentrations from 3 to 100 μM, *in vitro* and *in vivo*. This suggests that glycosylation at the C7 position is not favourable for an anti-angiogenic effect (Lam, Alex et al. 2012). However in another study, naringenin glucosides showed inhibitory angiogenic activity by blocking VEGF secretion, with naringenin being inactive (Schindler and Mentlein 2006).

The concentration of the flavonoids tested in these *in vivo* and *in vitro* assays were mostly at the level of 20-100 μM which is much higher than plasma flavonoid concentrations.

Table 5.1. Structure-activity relationship of some structural motifs in flavonoids.

Structural motifs	Anti-angiogenesis	Anti-proliferation	Anti-Inflammation	Anti-oxidant	Cytotoxicity
C2-C3 double bond conjugated with C4=O	Y (Fotsis, Pepper et al. 1997; Schindler and Mentlein 2006)	Y (Kawaii, Tomono et al. 1999; Rodriguez, Yanez et al. 2002; Comalada, Ballester et al. 2006)	Y (Comalada, Ballester et al. 2006)	Y (Ratty and Das 1988; Mathiesen, Malterud et al. 1997; Silva, Santos et al. 2002)	Y (Schindler and Mentlein 2006; Touil, Fellous et al. 2009)
C3′-OH,C4′-OH (catechol structure)	Y (Fotsis, Pepper et al. 1997)		Y (Comalada, Ballester et al. 2006)	Y (Cao, Sofic et al. 1997; Benavente-Garcia and Castillo 2008)	Y (Beutler, Hamel et al. 1998; Monasterio, Urdaci et al. 2004; Touil, Fellous et al. 2009)
C4′-OH			Y (Comalada, Ballester et al. 2006)	Y (Cao, Sofic et al. 1997; Benavente-Garcia and Castillo 2008)	Y (Touil, Fellous et al. 2009)
C3-OH	Y (Fotsis, Pepper et al. 1997)	NE (Kawaii, Tomono et al. 1999; Manthey and Guthrie 2002; Rodriguez, Yanez et al. 2002)	NE (Comalada, Ballester et al. 2006)	Y (Ratty and Das 1988; Burda and Oleszek 2001; Silva, Santos et al. 2002)	Y (Touil, Fellous et al. 2009)
C6-OH					Y (Touil, Fellous et al. 2009)
C3′-OCH_3	Y (Lam, Alex et al. 2012)			N (Dugas, Castaneda-Acosta et al. 2000; Burda and Oleszek 2001)	
C4′-OCH_3	Y (Lam, Alex et al. 2012)			N (Dugas, Castaneda-Acosta et al. 2000; Burda and Oleszek 2001)	
C5-OCH_3	Y (Lam, Alex et al. 2012)			N (Dugas, Castaneda-Acosta et al. 2000; Burda and Oleszek 2001)	
C6-OCH_3	Y (Lam, Alex et al. 2012)			N (Dugas, Castaneda-Acosta et al. 2000; Burda and Oleszek 2001)	
C7-OCH_3	Y (Lam, Alex et al. 2012)			N (Dugas, Castaneda-Acosta et al. 2000; Burda and Oleszek 2001)	
C8-OCH_3	Y (Lam, Alex et al. 2012)	Y (Kawaii, Tomono et al. 1999)			N (Dugas, Castaneda-Acosta et al. 2000; Monasterio, Urdaci et al. 2004; Lam, Alex et al. 2012)
Glycosylation at C7	N (Lam, Alex et al. 2012)				N (Touil, Fellous et al. 2009)

Flavonoids backbone and some structures are depicted in Figure 1.3 and Table 1.1. Y (positive effect), N (negative effect), NE (no effect)

5.2. Research aims

Flavonoids are well studied as anti-tumour agents. They are thought to act via their anti-angiogenic and anti-proliferative activities. However, most experiments have been performed *in vitro* and also with high concentrations of compounds. The concentrations used in these studies are not comparable with the *in vivo* conditions when one considers the bioavailability of flavonoids (after ingestion) in plasma and tissues. This chapter focuses on the angiogenic modulating activity of flavonoids at their likely *in vivo* concentrations as well as investigating the structure activity relationship to identify important structural motifs involved in angiogenesis inhibition/enhancement.

5.3. Research objectives

1) Identify pro-angiogenic flavonoids using rat aorta pro-angiogenesis bioassay

2) Investigate the pro-angiogenic activity of flavonoids at concentrations approximating human plasma concentrations to assess their possible positive influence on wound healing and cardiovascular disease.

3) Determine the structure-relationship activity including the effect of C2-C3 double bond, phenolic rings (A, B or C), glycosylation at C7, OH groups on A and B ring, and methoxylated OH groups on pro-angiogenic activity.

4) Determine the cellular mode of action of flavonoids, naringenin and genistein, with pro-angiogenic activity at concentrations close to *in vivo* human plasma levels.

5.4. Results

5.4.1. *In vitro* rat aorta pro-angiogenesis bioassay of flavonoids to screen for the most pro-angiogenic compounds

As described in chapter 4, the rat aorta angiogenesis bioassay has been used in the Parish laboratory to screen for compounds and other small molecules that could either enhance or inhibit the formation of new capillaries (Parish, Freeman et al. 1999). In order to screen for pro-angiogenic activity, the rat aorta ring bioassay was performed using medium containing 5% HIFCS (as in Chapter 4). These culture conditions resulted in sub-optimal vessel outgrowth (approximately 60% vessel occupancy after 7 days culture), which enabled both the stimulatory and inhibitory effects of test compounds on angiogenesis to be measured in the one assay. In this thesis the assay was quantified manually by measuring the relative density and the number of new vessels sprouted from the aorta ring in the field of view, which is represented as percentage growth.

To investigate the relation between the structure and pro-angiogenic activity of flavonoids, eighteen flavonoids were chosen based on structural features including, the substituent patterns of the A and C rings, the presence and absence of the C2-C3 double bond, the number and position of hydroxyl and methoxyl groups on the A and B ring (Table 5.2) and their demonstrated biological activity. These were then screened for activity with an *in vitro* rat aorta bioassay at 5×10^{-7} M and 5×10^{-8} M (Fig. 5.2). These concentrations were selected to represent the levels of flavonoids found in plasma and tissue (10^{-6} M to 10^{-9} M) following oral ingestion of a normal diet (Hollman 2004; Williamson and Manach 2005).

Seven flavonoids (genistein, 7-hydroxycoumarin, naringenin, dihydroquercetin, dihydrokaempferol, flavanone and naringenin-7-*O*-glucoside) approached significant pro-angiogenic activity at both 5×10^{-7} M and 5×10^{-8} M in more than three individual tests. They were chosen for further investigation. These flavonoids were then tested for pro-angiogenic activity in the presence of the endogenous growth factor bFGF at 12.5 ng/mL (as done in Chapter 4) and demonstrated similar enhancement in the growth of new capillaries at 5×10^{-7} M (Fig. 5.3) but only naringenin, dihydrokaempferol and

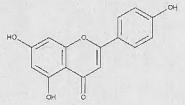
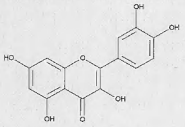
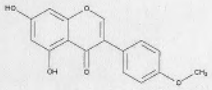
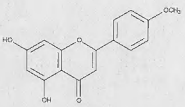
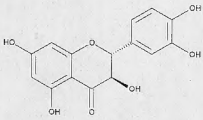
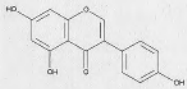
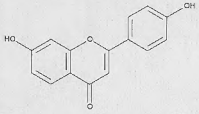
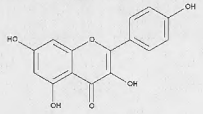
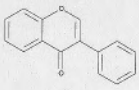
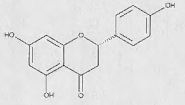
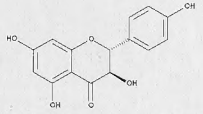
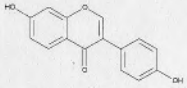
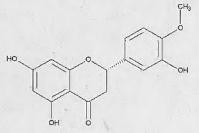
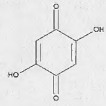
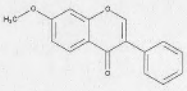
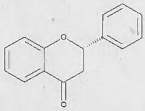
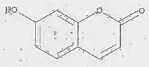
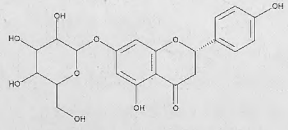
genistein approached significant enhancement in pro-angiogenic activity at 5×10^{-8} M (Fig. 5.4).

The anti-angiogenic activity of genistein (1-250 μM) and the anti-tumour effect of naringenin (10, 100 and 200 μM) have been reported previously (Fotsis, Pepper et al. 1995; Manthey and Guthrie 2002; Buchler, Reber et al. 2004; Sasamura, Takahashi et al. 2004; Zhang, Du et al. 2009). Therefore, genistein, naringenin and five other flavonoids were tested with in the *in vitro* pro-angiogenesis assay at 5×10^{-6} M (Fig. 5.5). No significant pro-angiogenic activity was observed for flavonoids tested at 5×10^{-6} M and interestingly genistein at 5×10^{-6} M inhibited the angiogenesis process although this did not reach significant levels (unpaired t-test, $P = 0.0998$ at day 7). However, in our hands, naringenin showed elevated pro-angiogenic response for 5×10^{-6} M in five out of seven tests and in two tests it reached significant levels.

In order to compare our findings to the literature, anti-angiogenic activity of genistein at 100 and 200 μM was assayed by the *in vitro* anti-angiogenesis rat aorta bioassay performed using medium containing 20% HIFCS (Fig. 5.6) and it demonstrated a very significant anti-angiogenic activity at both 100 and 200 μM with the stronger inhibition effect at 200 μM. Both genistein and naringenin were tested for pro-angiogenic activity by titrating the angiogenic effect at concentrations from 5×10^{-6} M to 5×10^{-12} M. Genistein, as expected, showed anti-angiogenic activity at the highest concentration tested (5×10^{-6} M) and demonstrated significant stimulation of angiogenesis at 5×10^{-7} M to 5×10^{-9} M (Fig. 5.7). Interestingly for naringenin, angiogenesis was stimulated at most concentrations tested from 5×10^{-6} M to 5×10^{-12} M and this effect reached the significant levels from 5×10^{-9} M and 5×10^{-10} M with maximal effect at 5×10^{-9} M, $p \leq 0.01$ (Fig. 5.8).

Seven chosen flavonoids and specifically genistein and naringenin were then selected to study the cellular mode of action.

Table 5.2. Chemical structures of eighteen flavonoids screened in the *in vitro* rat aorta ring pro-angiogenesis assay.

Flavones	Flavonols and Dihydroflavonols	Isoflavonoids
 Apigenin	 Quercetin	 Biochanin A
 Acacetin	 Dihydroquercetin (DHQ)	 Genistein
 4′,7-Dihydroxyflavone (DHF)	 Kaempferol	 Isoflavone
Flavanone		
 Naringenin	 Dihydrokaempferol (DHK)	 Daidzein
	Others	
 Hesperetin	 2,5-Dihydroxy-1,4-benzoquinone	 7-Methoxyisoflavone
 Flavanone	 7-Hydroxycoumarin	
 Naringenin-7-*O*-glucoside		

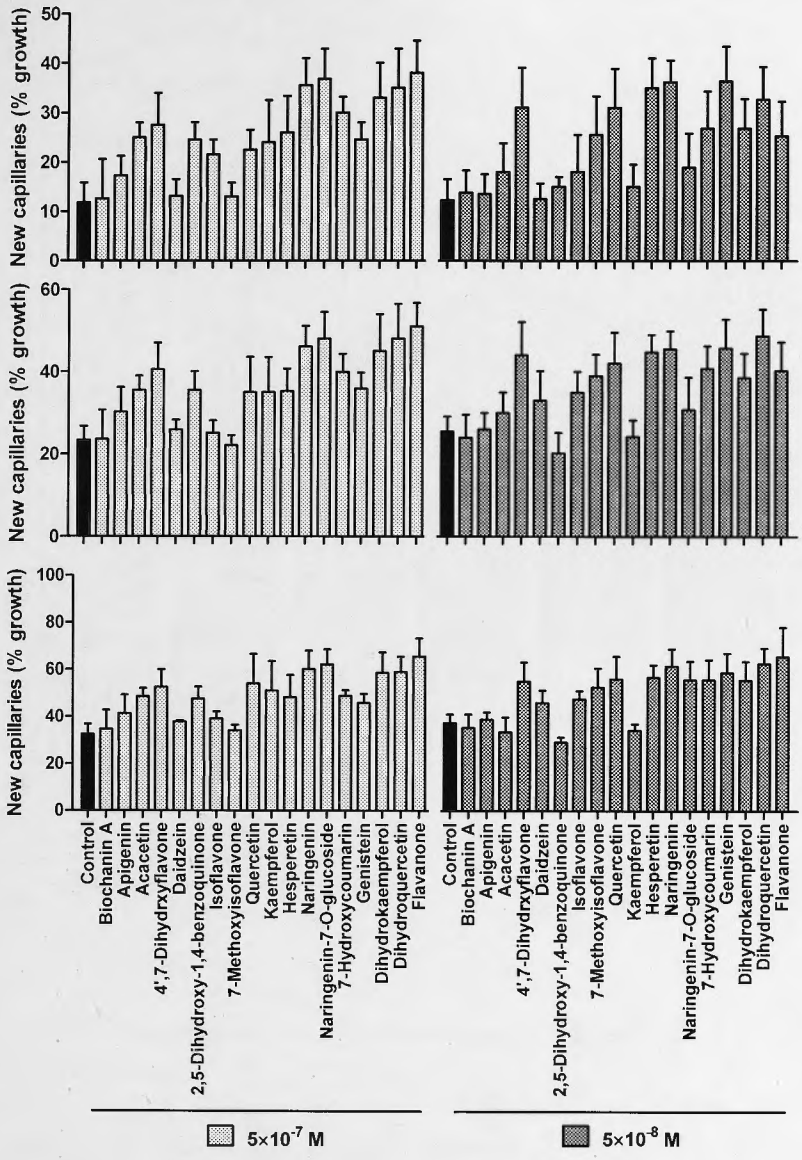


Figure 5.2. Screening for pro-angiogenic activity of flavonoids in the *in vitro* rat aorta assay.

Structure pro-angiogenic activity relationship of eighteen flavonoids was screened with an *in vitro* rat aorta bioassay at concentrations of 5×10^{-7} and 5×10^{-8} M. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5 (A), 6 (B) and 7 (C). Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). Two-way ANOVA analysis was performed comparing 5×10^{-7} M and 5×10^{-8} M concentration on day 5, 6 and 7. No significant difference was observed.

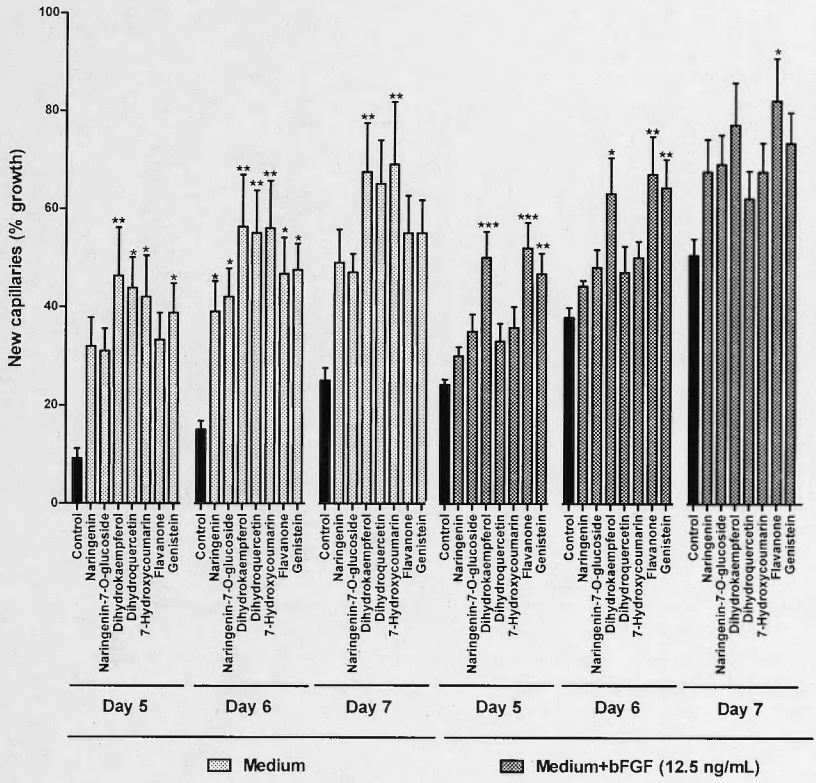


Figure 5.3. Pro-angiogenic activity of flavonoids at 5×10^{-7} M ± bFGF in the *in vitro* rat aorta assay.

Effect of seven chosen flavonoids was tested at 5×10^{-7} M in pro-angiogenesis medium, M199 supplemented with 5% HIFCS (medium) with and without the mitogen bFGF (12.5 ng/mL). Control cultures contained the same diluent dilution as the test compounds. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5, 6 and 7. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01, ***, ≤ 0.001. No significant effect was observed using two-way ANOVA analysis between compounds treated with medium and medium+bFGF (12.5 ng/mL) treatments on day 5, 6 and 7.

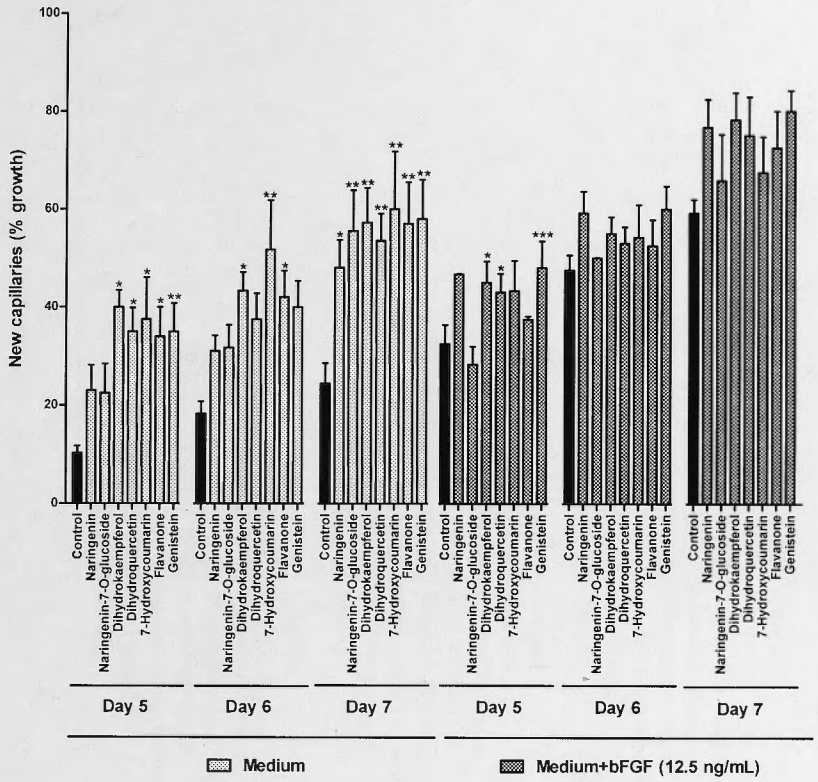


Figure 5.4. Pro-angiogenic activity of flavonoids at 5×10^{-8} M ± bFGF in the *in vitro* rat aorta assay.

Effect of seven chosen flavonoids was tested at 5×10^{-8} M in pro-angiogenesis medium, M199 supplemented with 5% HIFCS (medium) with and without the mitogen bFGF (12.5 ng/mL). Control cultures contained the same diluent dilution as the test compounds. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5, 6 and 7. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01, ***, ≤ 0.001. Two-way ANOVA analysis was performed comparing medium and medium+bFGF (12.5 ng/mL) treated compounds on day 5, 6 and 7 and only naringenin showed significant difference on days 6 and 7, $P \leq 0.05$.

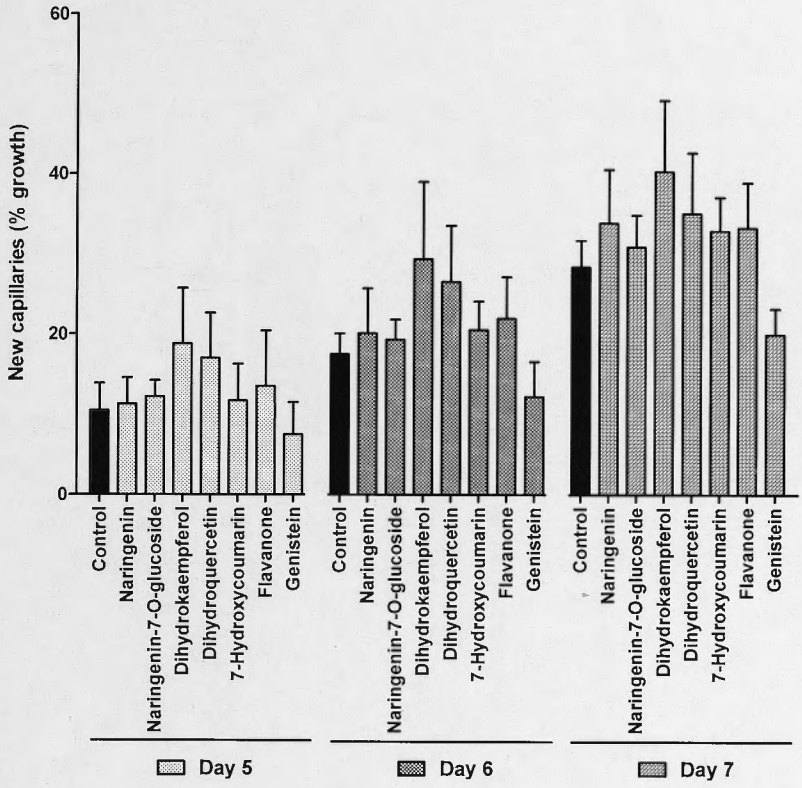


Figure 5.5. Pro-angiogenic activity of flavonoids at 5×10^{-6} M in the *in vitro* rat aorta assay.

Effect of seven chosen flavonoids was tested at 5×10^{-6} M in pro-angiogenesis medium, M199 supplemented with 5% HIFCS. Control cultures contained the same diluent dilution as the test compounds. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5, 6 and 7. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). No significant effect was observed.

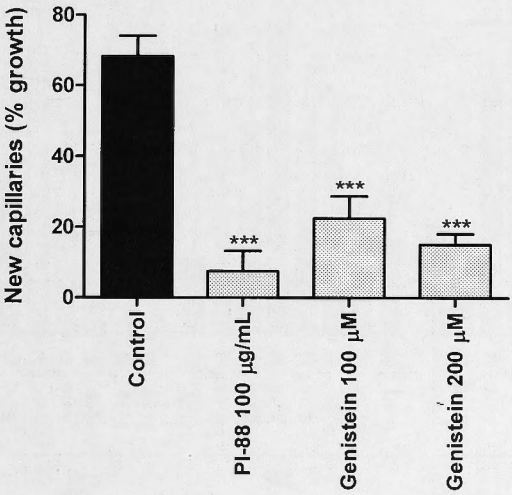


Figure 5.6. Anti-angiogenic activity of genistein at 100 and 200 µM in the *in vitro* rat aorta assay.

Effect of genistein was tested at 100×10^{-6} M and 200×10^{-6} M in anti-angiogenesis medium, M199 supplemented with 20% HIFCS. PI-88 has clinically proven anti-angiogenic activity and was used as a positive control. Control cultures contained the same diluent dilution as the test compounds. Data are expressed as a percentage of aorta vessel outgrowths observed on day 5. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. ***, $P \leq 0.001$.

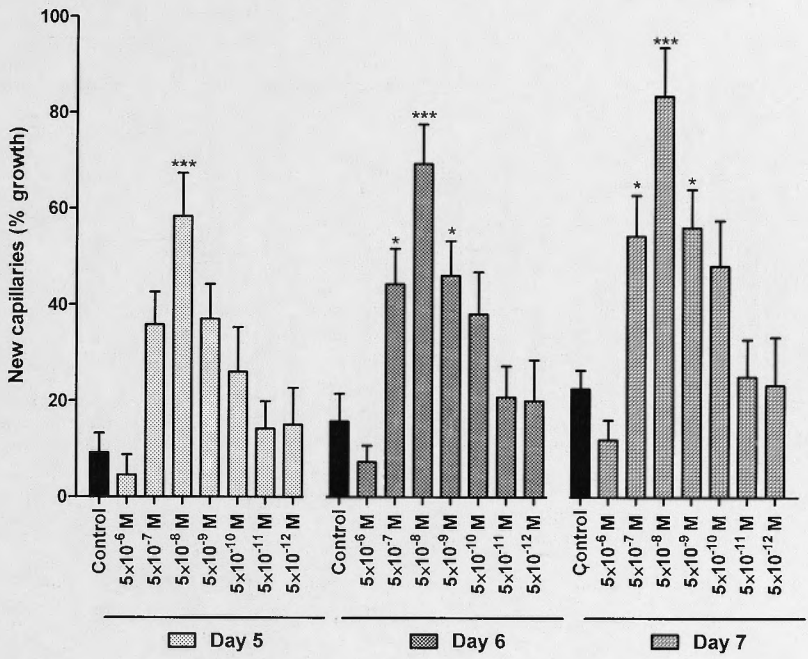


Figure 5,7. Pro-angiogenic activity of genistein at 5×10^{-6} M to 5×10^{-12} M in the *in vitro* rat aorta assay.

Effect of genistein was tested at 5×10^{-6} M to 5×10^{-12} M, in pro-angiogenesis medium, M199 supplemented with 5% HIFCS. Control cultures contained the same diluent dilution as the test compounds. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5, 6 and 7. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01, ***, $P \leq 0.001$.

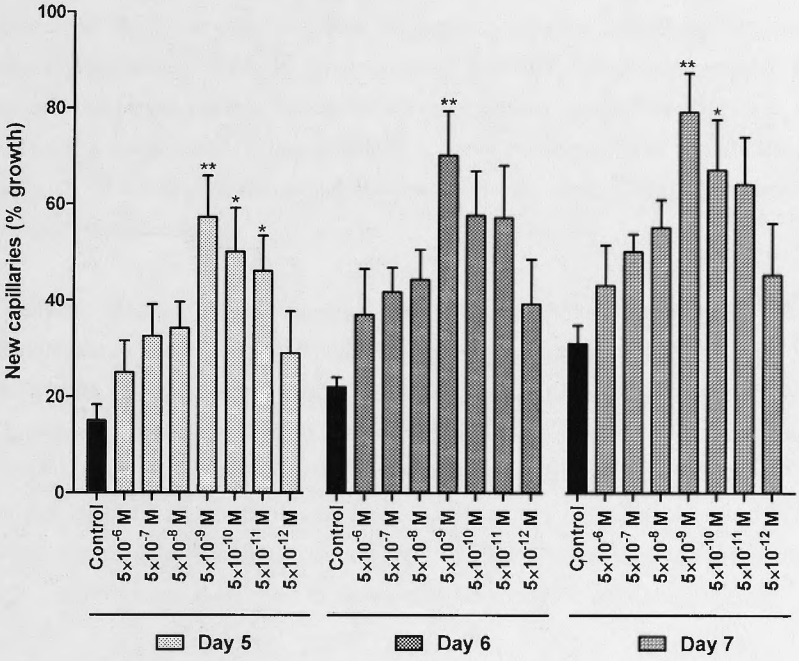


Figure 5.8. Pro-angiogenic activity of naringenin at 5×10^{-6} M to 5×10^{-12} M in the *in vitro* rat aorta assay.

Effect of naringenin was tested at 5×10^{-6} M to 5×10^{-12} M, in pro-angiogenesis medium, M199 supplemented with 5% HIFCS. Control cultures contained the same diluent dilution as the test compounds. Data are expressed as a percentage of aorta vessel outgrowths observed on days 5, 6 and 7. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01.

5.4.2. Effects of chosen flavonoids on endothelial cell proliferation

In order to determine the effect of the seven chosen flavonoids and in particular naringenin and genistein, on cell proliferation, *in vitro* HUVEC proliferation assays were employed based on ^{3}H-thymidine incorporation. In some of the proliferation experiments bFGF was added to assess whether or not the test compounds influenced the mitogenic activity of bFGF. Optimisation of the bFGF concentration required for the proliferation experiments was carried out on serum-starved confluent HUVEC. As explained in chapter 4, a 12.5 ng/mL bFGF concentration appeared to be sufficient to induce HUVEC cell proliferation and was optimal for detecting enhanced proliferation by the test compounds.

Initially, all seven chosen flavonoids were tested at 5×10^{-7} M and 5×10^{-8} M ± bFGF for their effect on the proliferation of serum-starved confluent HUVEC (Fig. 5.9). The results showed that in the absence of bFGF, naringenin (5×10^{-7} M), dihydrokaempferol (5×10^{-8} M), dihydroquercetin (5×10^{-7} M), 7-hydroxycoumarin (5×10^{-8} M) and flavanone (5×10^{-8} M) could enhanced cell proliferation. The introduction of bFGF into the system at a concentration of 12.5 ng/mL, resulted in an overall increase in cell proliferation. However, significant cell proliferation beyond that of the control was again observed for naringenin, 7-hydroxycoumarin and flavanone.

Further experiments were performed using naringenin and genistein at 5×10^{-6} M to 5×10^{-12} M using serum-starved confluent HUVEC ± bFGF (12.5 ng/mL). These experiments confirmed the results from the initial experiments for naringenin and genistein. A much stronger proliferative response was observed with naringenin and it reached significant levels in the absence (5×10^{-6}, 5×10^{-7}, 5×10^{-8} and 5×10^{-9} M) and presence of bFGF (5×10^{-6} and 5×10^{-7}) (Fig. 5.10). These activities are comparable with the elevated pro-angiogenic activity of naringenin in the *in vitro* rat aorta assay. The results indicated that genistein in the absence of bFGF, at 5×10^{-7} M and 5×10^{-8} M did not significantly enhance cell proliferation. Interestingly, approached and reached statistically significant cell proliferation inhibition was observed at 5×10^{-6} M in the absence and presence of bFGF, respectively. The inhibitory effect did not reach significance for the rest of the genistein concentrations tested in the absence and presence of bFGF (Fig. 5.11).

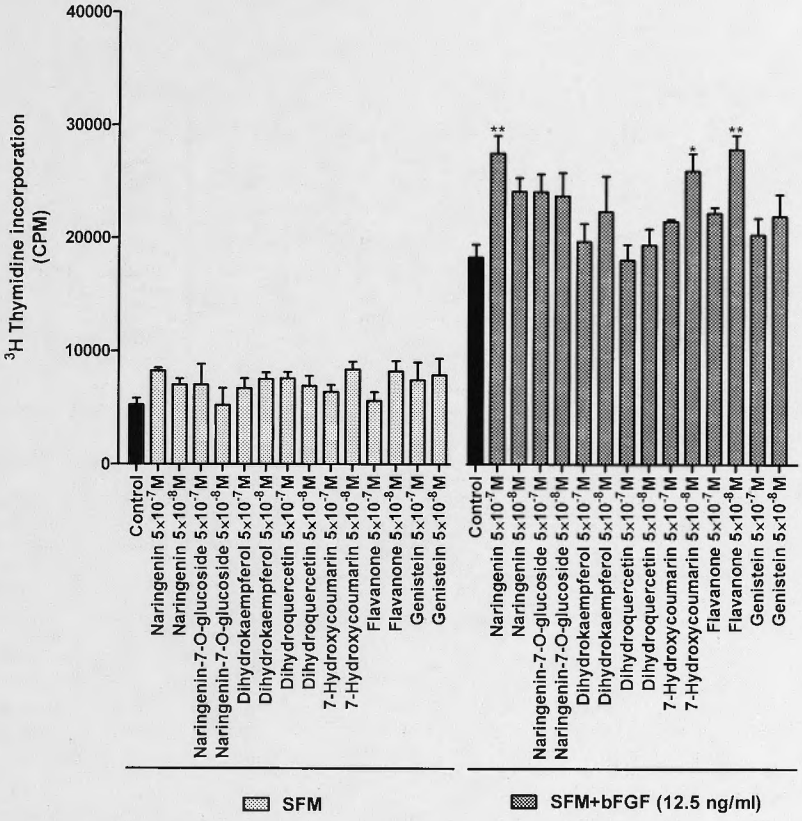


Figure 5.9. HUVEC proliferation assay of chosen flavonoids at 5×10^{-7} M and 5×10^{-8} M.

Effect of seven chosen flavonoids at 5×10^{-7} M and 5×10^{-8} M concentrations on confluent serum-starved HUVEC proliferation in serum free media (SFM) with and without the mitogen bFGF (12.5 ng/mL). Control cultures contained the same diluent dilution as the test compounds. Cell proliferation was measured as ^{3}H thymidine incorporation after 24 hours incubation. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01. A two-way ANOVA data analysis showed significant difference for all tested concentrations comparing their effect in SFM to SFM+bFGF (12.5 ng/mL) treatments, $P \leq 0.0001$.

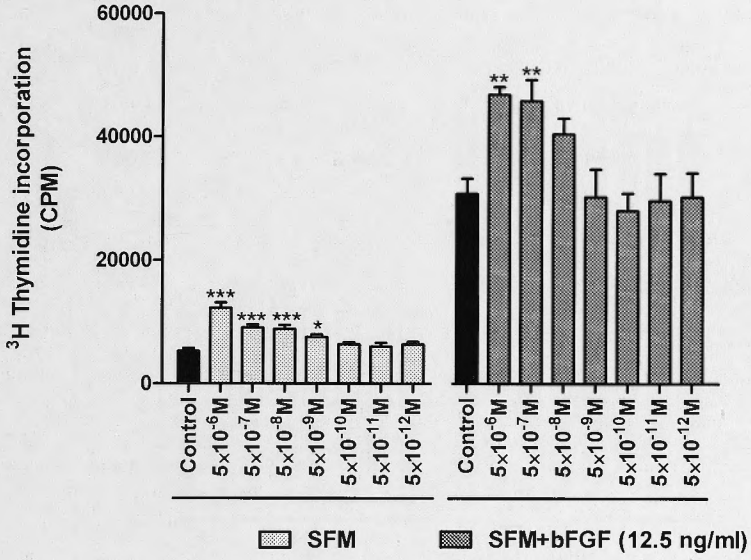


Figure 5.10. HUVEC proliferation assay in the presence of naringenin.

Effect of naringenin at concentrations from 5×10^{-6} M to 5×10^{-12} M, on confluent serum-starved HUVEC proliferation in serum free media (SFM) with and without the mitogen bFGF (12.5 ng/mL). Control cultures contained the same diluent dilution as the test compounds. Cell proliferation was measured as ^{3}H thymidine incorporation after 24 hours incubation. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01. A two-way ANOVA data analysis showed significant difference for all tested concentrations comparing their effect in SFM to SFM+bFGF (12.5 ng/mL) treatments, $P \leq 0.0001$.

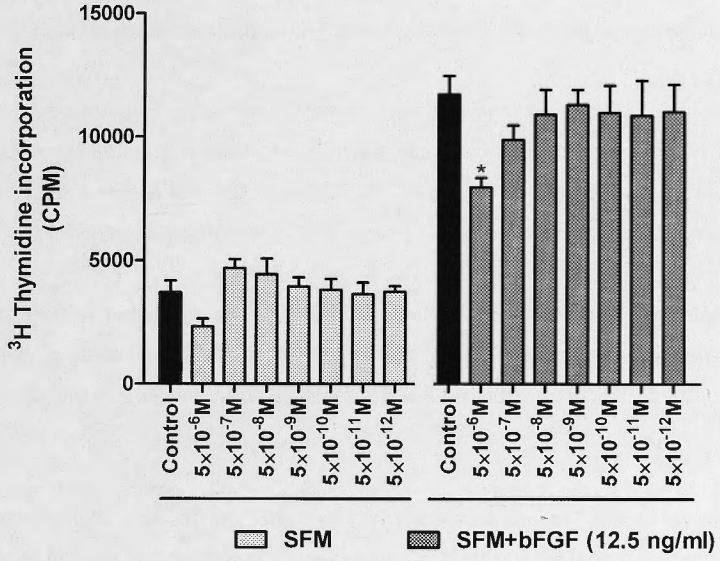


Figure 5.11. HUVEC proliferation assay in the presence of genistein.

Effect of genistein at concentrations from 5×10^{-6} M to 5×10^{-12} M, on confluent serum-starved HUVEC proliferation in serum free media (SFM) with and without the mitogen bFGF (12.5 ng/mL). Control cultures contained the same diluent dilution as the test compounds. Cell proliferation was measured as ^{3}H thymidine incorporation after 24 hours incubation. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$. A two-way ANOVA data analysis showed significant difference for all tested concentrations comparing their effect in SFM to SFM+bFGF (12.5 ng/mL) treatments, $P \leq 0.0001$.

5.4.3. Effect of chosen flavonoids on endothelial cell migration (wound healing assay)

As mentioned in chapter 4, cell migration was determined using a "wound healing" assay. The effect of the test compounds on the recovering wound was measured at 2 hour intervals with confluent monolayers of HMEC in 96-well plates. The results were then expressed as percentage wound confluence relative to the initial wound width.

The application of the chosen flavonoids, naringenin-7-*O*-glucoside, dihydrokaempferol, dihydroquercetin, 7-hydroxycoumarin and flavanone at 5×10^{-6} M (Fig. 5.12), 5×10^{-7} M (Fig. 5.13) and 5×10^{-8} M (Fig. 5.14) concentrations, made no significant difference to the rate of HMEC wound healing compared to control cultures.

The effect of naringenin and genistein on HMEC wound recovery was determined separately at concentrations from 5×10^{-6} M to 5×10^{-12} M. Again no statistically significant effect on wound recovery was observed for either naringenin (Fig. 5.15) or genistein (Fig. 5.16).

In conclusion, none of the flavonoids, at the tested coneentrations, exerted any significant influence on the rate of wound healing of HMEC monolayer.

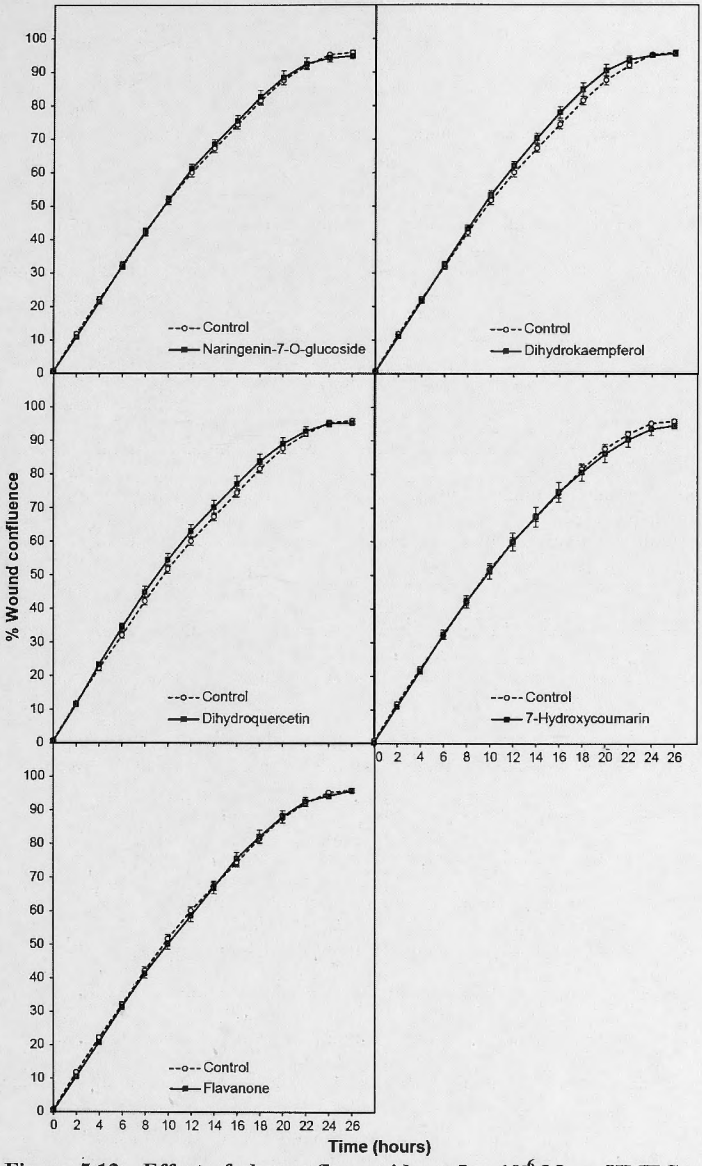


Figure 5.12. Effect of chosen flavonoids at 5×10^{-6} M on HMEC wound healing assay.

Effect of chosen flavonoids at 5×10^{-6} M on wound recovery was measured as % wound confluence from 0 to 26 hours relative to the initial wound marks. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=12). No significant difference in cell adhesion was observed.

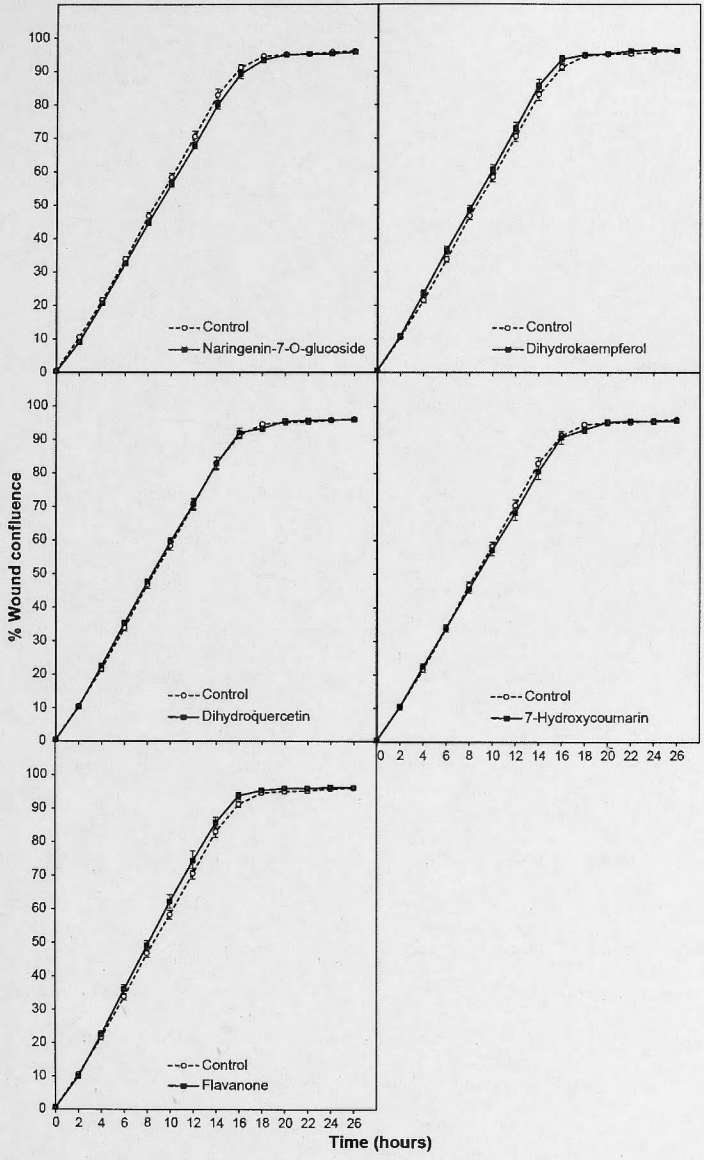


Figure 5.13. Effect of chosen flavonoids at 5×10^{-7} M on HMEC wound healing assay.

Effect of chosen flavonoids at 5×10^{-7} M on wound recovery was measured as % wound confluence from 0 to 26 hours relative to the initial wound marks. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=12). No significant difference in cell adhesion was observed.

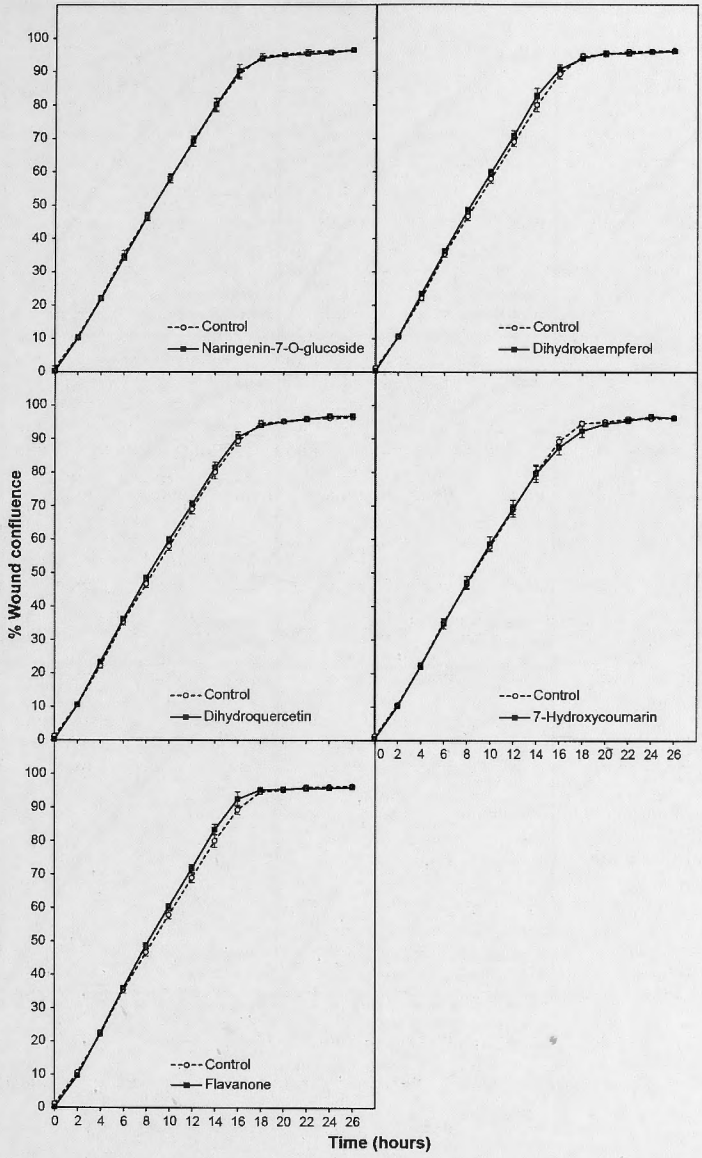


Figure 5.14. Effect of chosen flavonoids at 5×10^{-8} M on HMEC wound healing assay.

Effect of chosen flavonoids at 5×10^{-8} M on wound recovery was measured as % wound confluence from 0 to 26 hours relative to the initial wound marks. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=12). No significant difference in cell adhesion was observed.

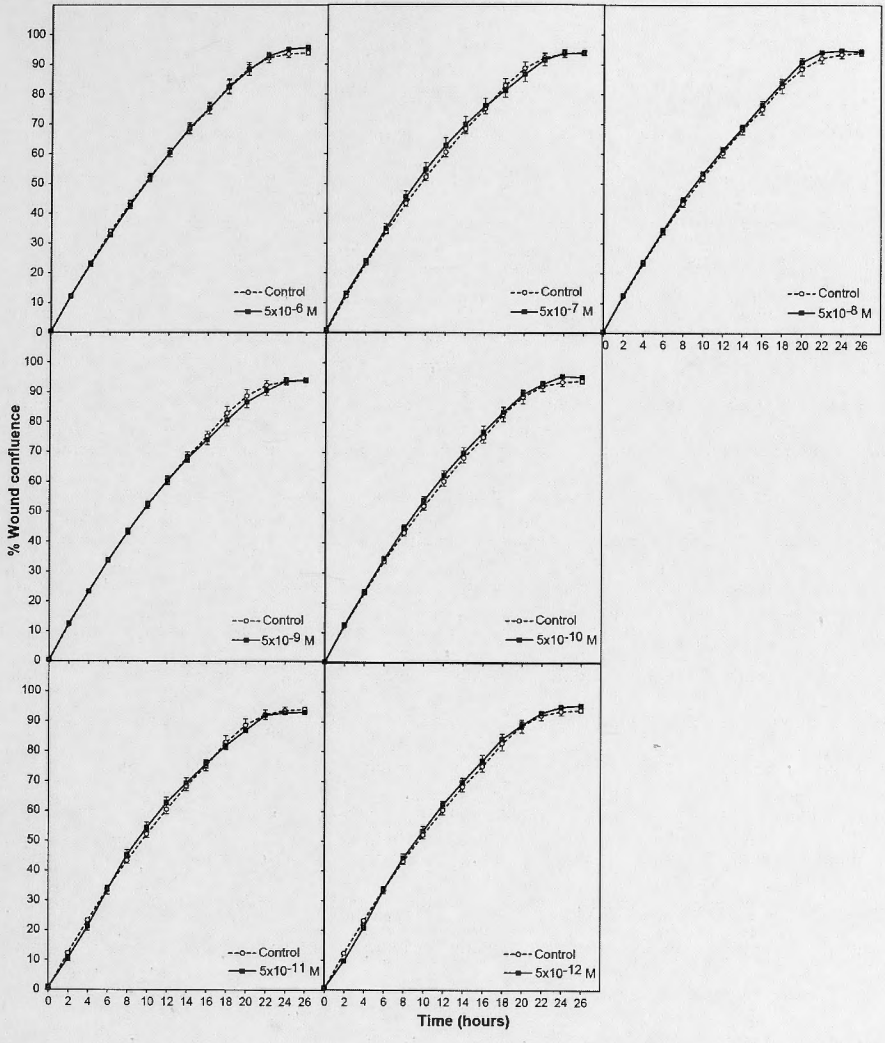


Figure 5.15. Effect of naringenin on HMEC wound healing assay.

Effect of naringenin at concentrations from 5×10^{-6} M to 5×10^{-12} M on wound recovery was measured as % wound confluence from 0 to 26 hours relative to the initial wound marks. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=12). No significant difference in cell adhesion was observed.

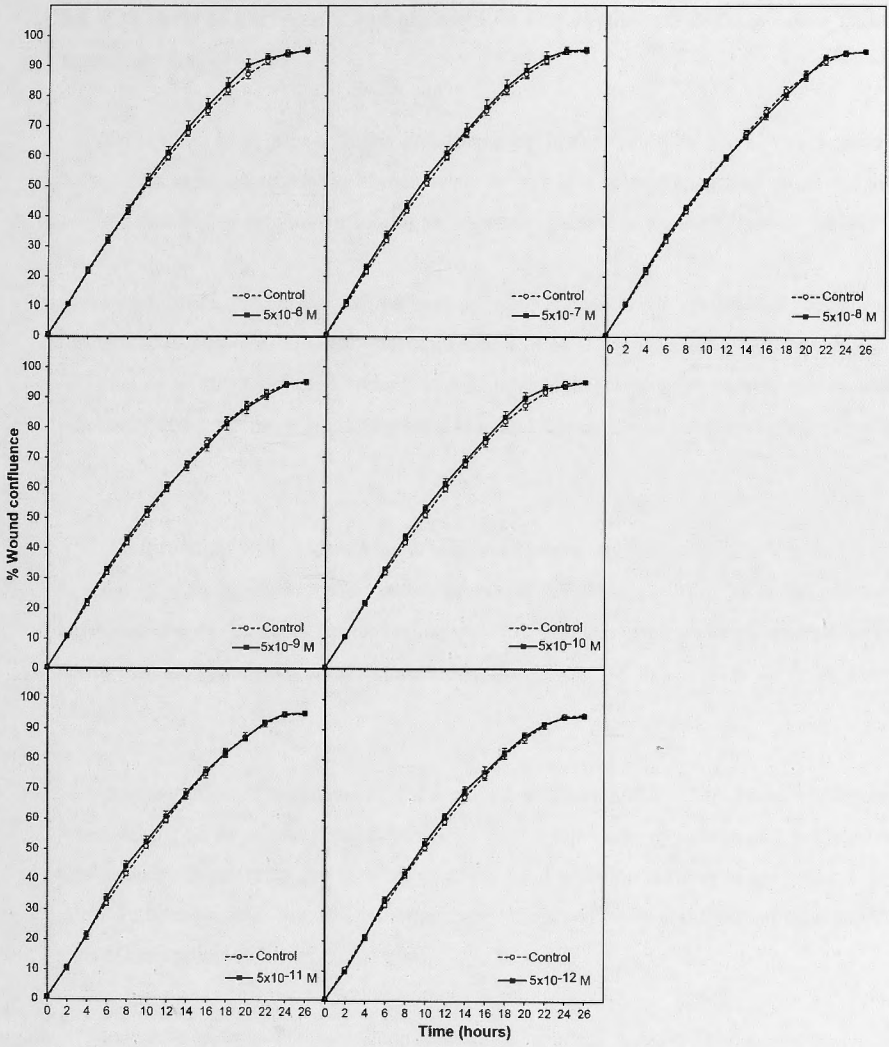


Figure 5.16. Effect of genistein on HMEC wound healing assay.

Effect of genistein at concentrations from 5×10^{-6} M to 5×10^{-12} M on wound recovery was measured as % wound confluence from 0 to 26 hours relative to the initial wound marks. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=12). No significant difference in cell adhesion was observed.

5.4.4. Effects of naringenin and genistein on endothelial cell differentiation (tube formation assay)

As described in chapter 4, the *in vitro* formation of capillary-like tubes by endothelial cells on an appropriate basement membrane matrix is a powerful method to screen for various factors that promote or inhibit angiogenesis (Arnaoutova and Kleinman 2010).

To determine the effect of naringenin and genistein on endothelial cell tube formation, *in vitro* HUVEC tube formation assays on a Matrigel plug were employed. HUVEC (4×10^4 cells/well) were cultured on a Matrigel plug in 96 well plates and incubated at 37 °C in an IncuCyte incubator and observations were recorded every 2 hours.

Tube formation was evident after 2 hours of culture, and became complete in 12-18 hours (Fig. 4.4). Cells lost their connections and started to die after 18 hours culture. Different stages in tube formation in control cultures and cultures treated with naringenin and genistein at concentrations from 5×10^{-6} M to 5×10^{-12} M were compared.

Semi-quantitative analysis using the IncuCyte software and the NIH ImageJ software was established to measure the capacity of test compounds to induce or inhibit tube formation by quantifying the percentage of denuded area, the number of sprouting cells at earlier time points (4 hours incubation), the number of tubes and the total tube length at later time points (6 hours incubation).

The results showed that naringenin could enhance tube formation at a concentration of 5×10^{-7} M for all the characteristics tested but no significant effect was observed for the other tested concentrations (Fig. 5.17 and 5.18). Interestingly, genistein inhibited tube formation at 5×10^{-6} M by significantly reducing the number of tubes and sprouts, denuded area percentage (Fig. 5.19 and 5.20).

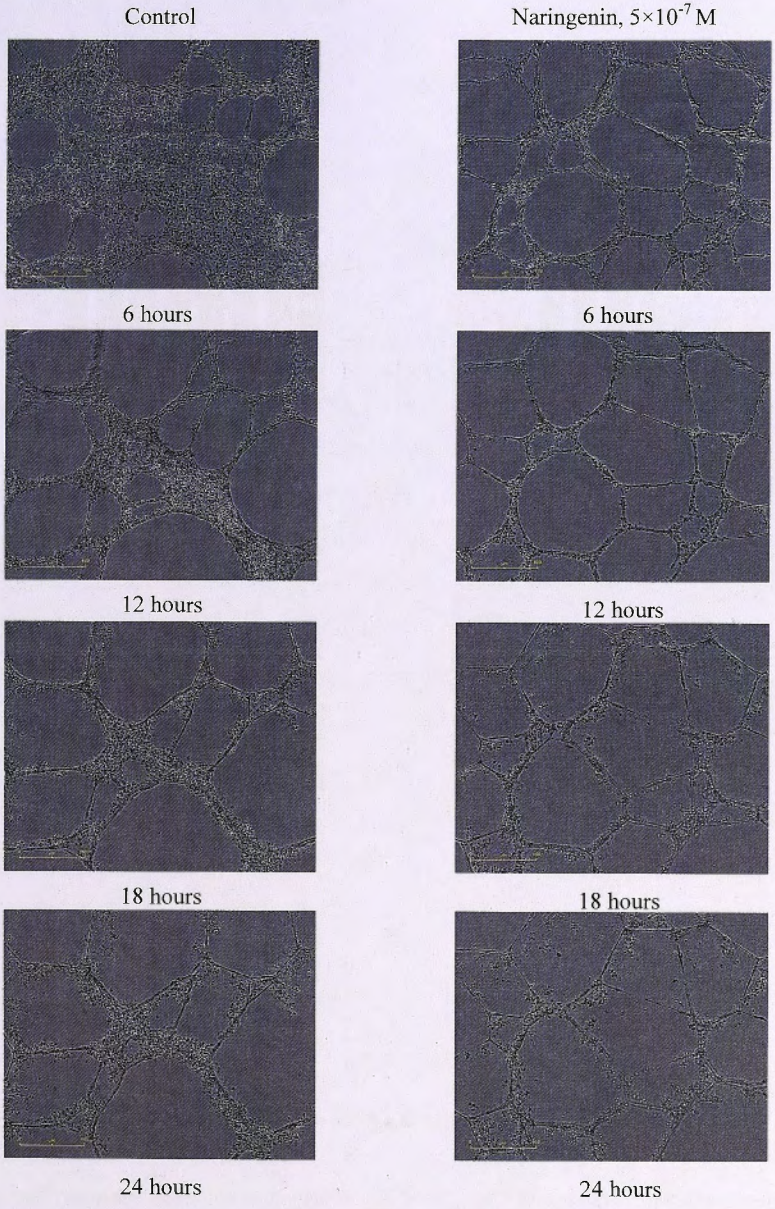


Figure 5.17. Effect of naringenin on HUVEC tube formation assay on Matrigel.

Effect of naringenin, at a concentration of 5×10^{-7} M on HUVEC tube formation was observed from 0 to 24 hours. Control cultures contained the same diluent dilution as the test compounds.

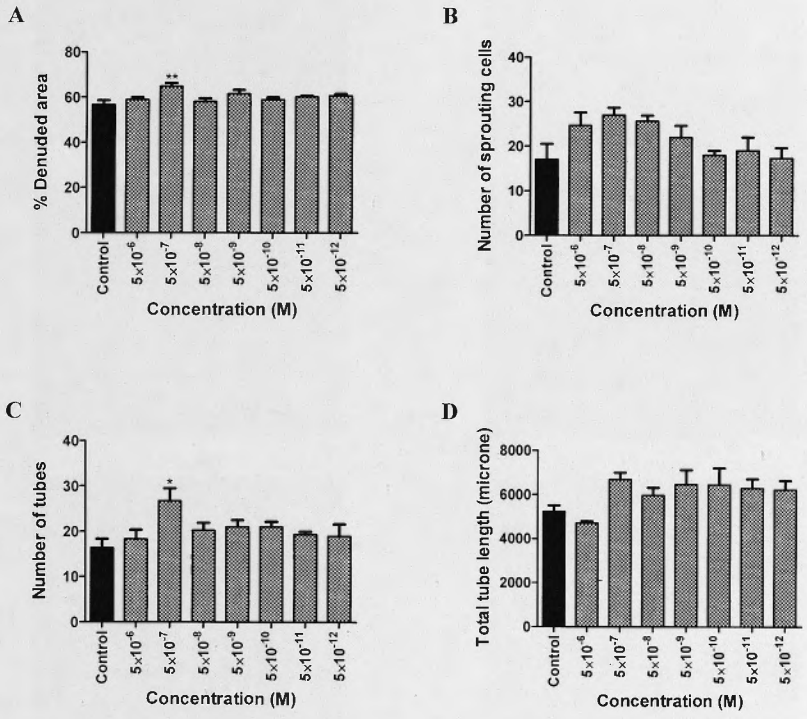


Figure 5.18. Effect of naringenin on HUVEC tube formation assay on Matrigel.

Effect of naringenin, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on HUVEC tube formation was measured, as percentage denuded area (A), number of sprouting cells (B), number of tubes (C) and total tube length (D). Parameters shown in (A) and (B) were measured after 4 hours culture and in (C) and (D) after 6 hours culture. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01.

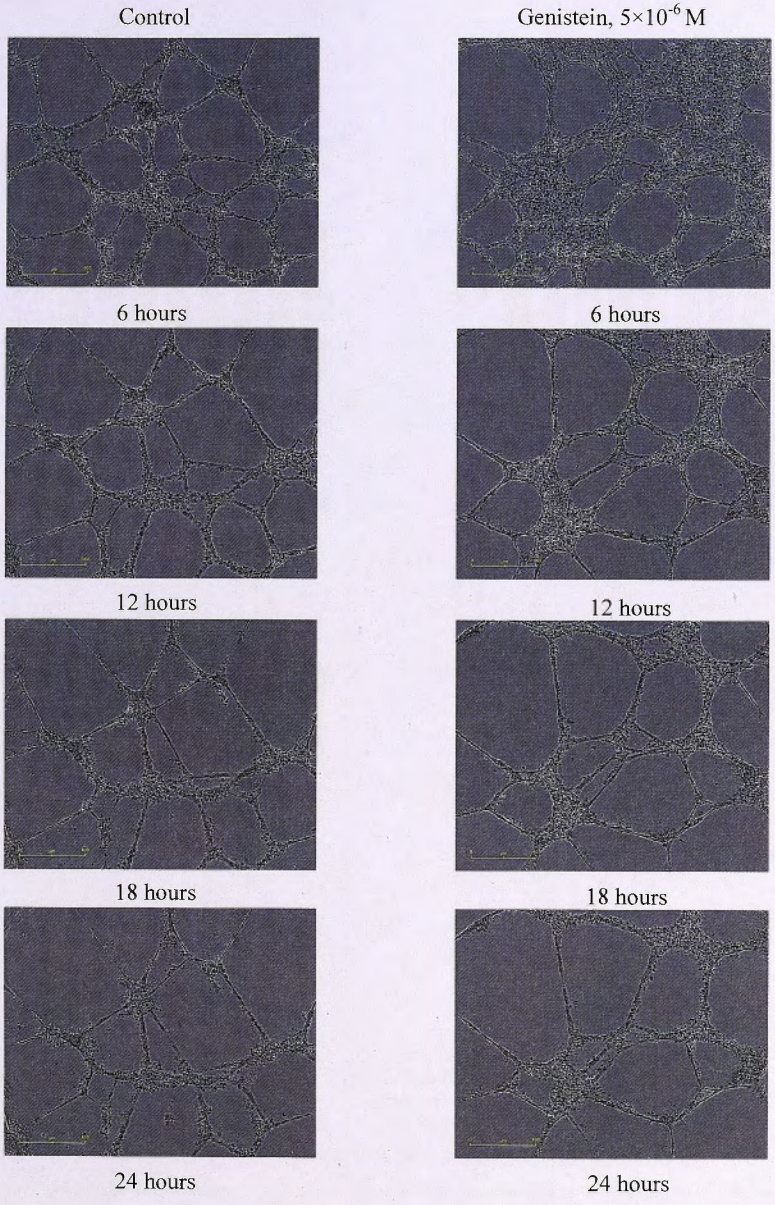


Figure 5.19. Effect of genistein on HUVEC tube formation assay on Matrigel.

Effect of genistein, at a concentration of 5×10^{-6} M on HUVEC tube formation was observed from 0 to 24 hours. Control cultures contained the same diluent dilution as the test compounds.

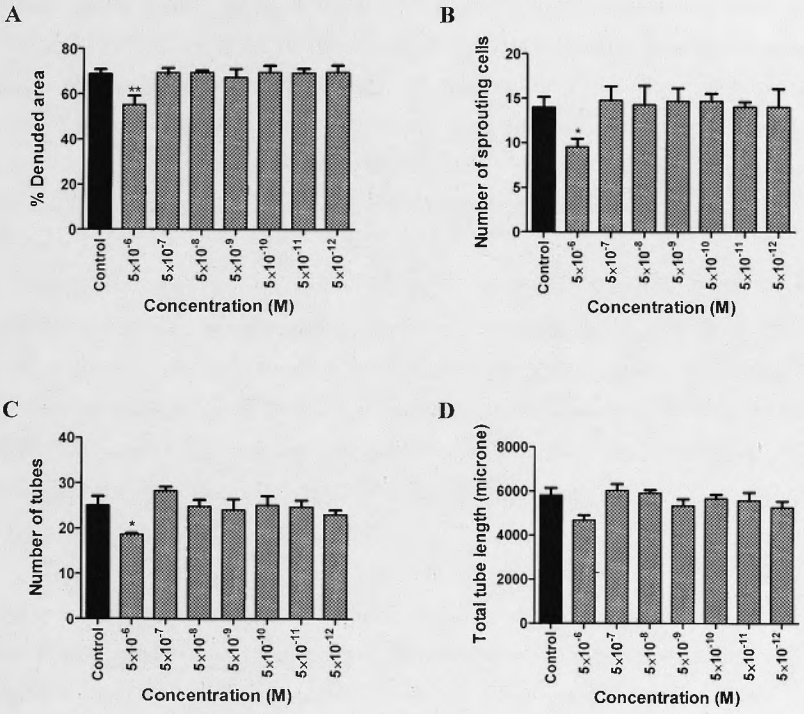


Figure 5.20. Effect of genistein on HUVEC tube formation assay on Matrigel.

Effect of genistein, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on HUVEC tube formation was measured, as percentage denuded area (A), number of sprouting cells (B), number of tubes (C) and total tube length (D). Parameters shown in (A) and (B) were measured after 4 hours culture and in (C) and (D) after 6 hours culture. Control cultures contained the same diluent dilution as the test compounds. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). *, $P \leq 0.05$, **, ≤ 0.01.

5.4.5. Effect of naringenin and genistein on endothelial cell adhesion (Rose Bengal assay)

As described in chapter 4, cell adhesion to ECM components fibronectin and vitronectin was determined by using a Rose Bengal adhesion assay and endothelial cells (HMEC). Optimisation of the fibronectin and vitronectin concentrations required to coat microwells for maximal cell adhesion are depicted in Fig. 4.11. The coating concentrations of fibronectin and vitronectin were established as 10 and 5 μg/mL, respectively, where sub-optimal cell binding was observed. These conditions are optimal for detecting either enhancement or inhibition of cell adhesion.

The ability of naringenin and genistein to affect the adhesion of HMEC to immobilised fibronectin was tested at concentrations ranging from 5×10^{-6} M to 5×10^{-12} M, after 30 minute and 60 minute incubations. The results showed that naringenin had no significant effect on HMEC binding to fibronectin after either 30 or 60 minutes incubation (Fig. 5.21). Similar cell adhesion experiments were performed using genistein. Genistein showed a slight effect in cell binding only at 5×10^{-7} M and after 60 minutes incubation (Fig. 5.22).

Experiments using synthetic ***FK1*** in the HMEC adhesion assay in chapter 4 showed that HMEC adhere much more slowly to immobilised vitronectin than fibronectin. Therefore, monitoring the first 30 minutes of cell-vitronectin binding when cell attachment occurs is very critical. Thus, the experiments were performed based on a time course covering 2.5 to 25 minutes. The results demonstrated that both naringenin (Fig. 5.23) and genistein (Fig. 5.24) had no significant effect on adhesion of HMEC to vitronectin.

Overall, the results demonstrated that the pro-angiogenic activity of naringenin (5×10^{-6} M to 5×10^{-12} M) and genistein (5×10^{-7} M to 5×10^{-12} M) and the anti-angiogenic activity of genistein (5×10^{-6} M) were essentially unconnected to the adhesion of HMEC to fibronectin and vitronectin.

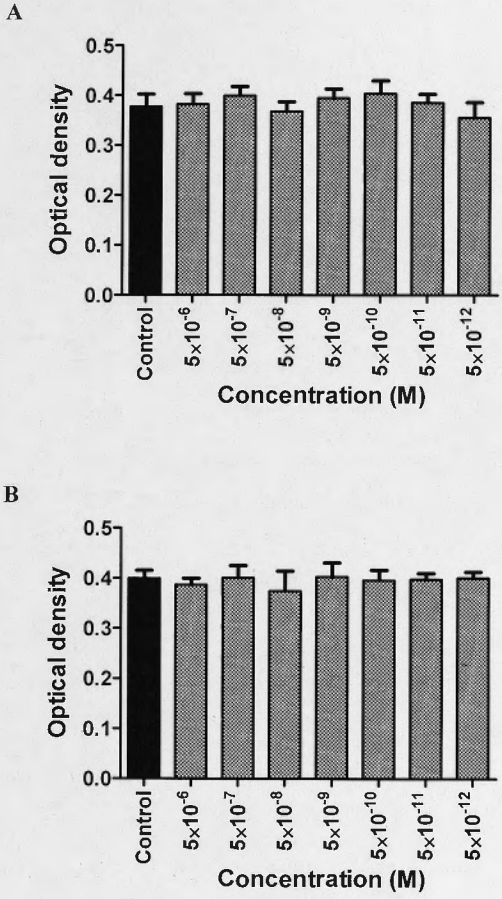


Figure 5.21. Effect of naringenin on fibronectin-mediated HMEC cell adhesion.

Effect of naringenin at concentrations from 5×10^{-6} M to 5×10^{-12} M, on cell adhesion was measured atfter 30 minutes (A) and 60 minutes (B) incubation as optical density of Rose Bengal staining of adherent cells. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). No significant difference in cell adhesion was observed.

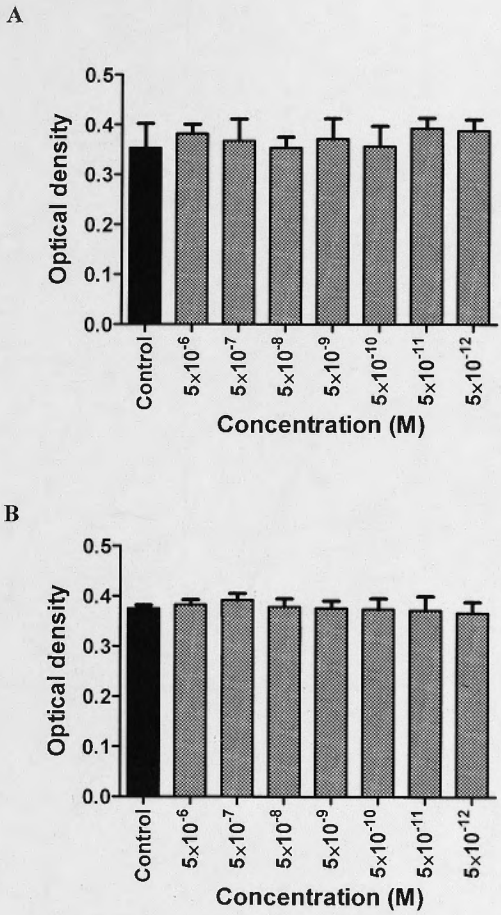


Figure 5.22. Effect of genistein on fibronectin-mediated HMEC cell adhesion.

Effect of genistein at concentrations from 5×10^{-6} M to 5×10^{-12} M on cell adhesion was measured after 30 minutes (A) and 60 minutes (B) incubation as optical density of Rose Bengal staining of adherent cells. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). No significant difference in cell adhesion was observed.

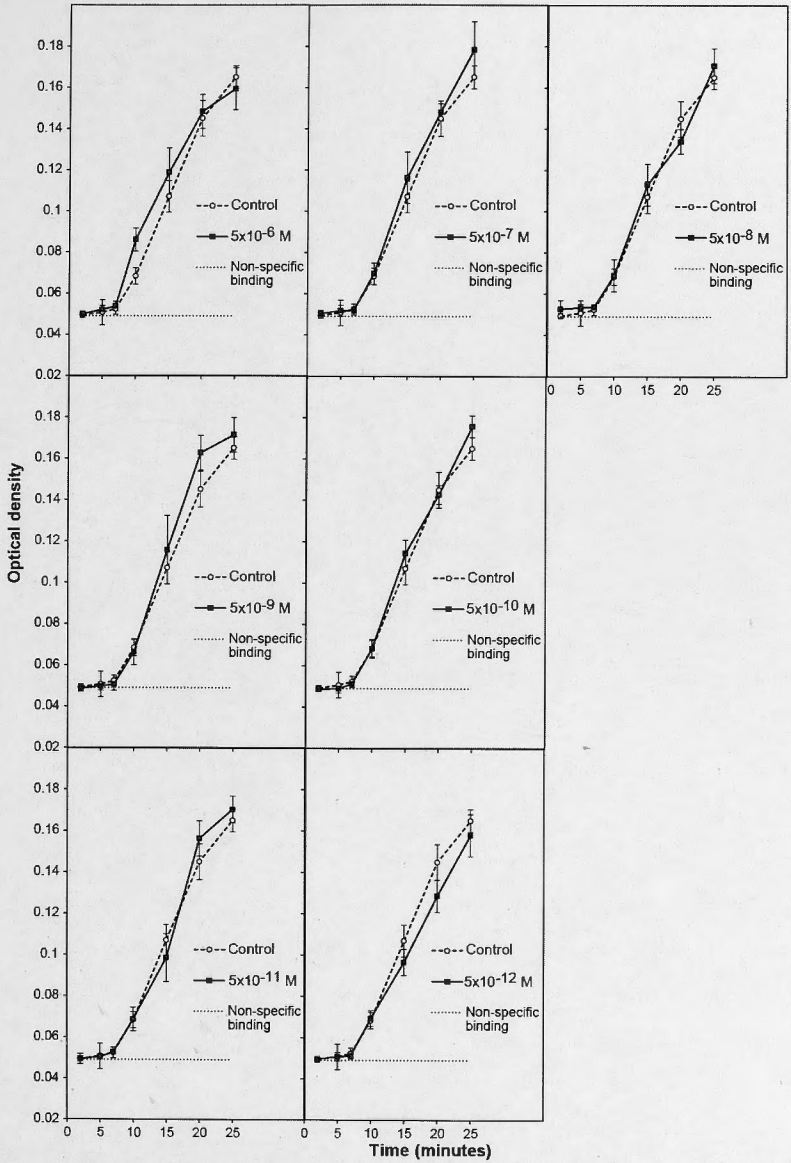


Figure 5.23. Time course of effect of naringenin on vitronectin-mediated HMEC cell adhesion.

Effect of naringenin, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on cell adhesion was measured, using plates coated with 5 µg/mL vitronectin and following 2.5-25 minute incubation, as optical density of Rose Bengal staining of adherent cells. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=4). No significant difference in cell adhesion was observed.

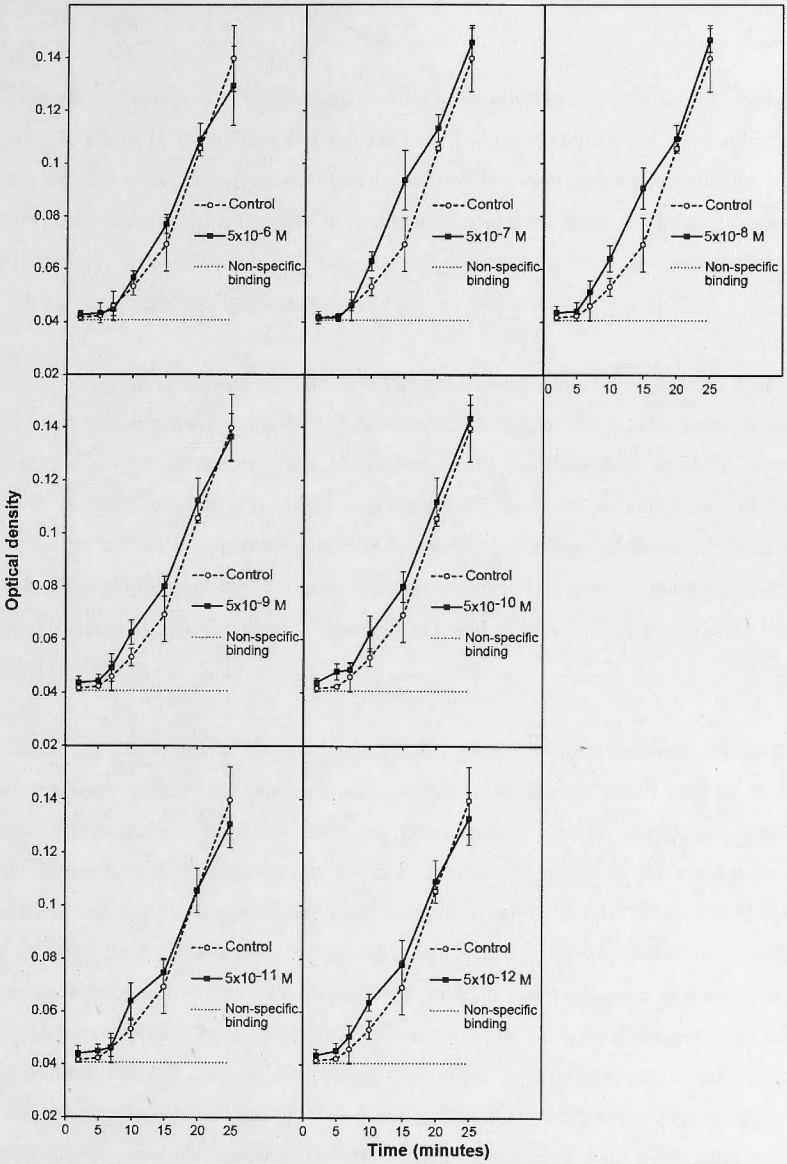


Figure 5.24. Time course of effect of genistein on vitronectin-mediated HMEC cell adhesion.

Effect of genistein, at concentrations from 5×10^{-6} M to 5×10^{-12} M, on cell adhesion was measured, using plates coated with 5 μg/mL vitronectin and following 2.5-25 minute incubation, as optical density of Rose Bengal staining of adherent cells. Data analysis was performed by one-way and two-way ANOVA, comparing each group to control and other groups. Error bars represent SEM (n=4). No significant difference in cell adhesion was observed.

5.5. Discussion

Flavonoids belong to a large family of natural polyphenolic compounds that are commonly found in the human diet especially in fruits, vegetables and beverages such as tea and red wine (Aherne and O'Brien 2002). Flavonoids have been studied for a variety of biological activities in humans including their anti-allergic, anti-inflammatory, anti-microbial and anti-oxidant activities as well as their preventive effects on cancer and cardiovascular disease (Ren, Qiao et al. 2003).

Compounds used to treat cancer are usually designed either to be cytotoxic or are directed at starving the tumour of its blood supply through their anti-vascular or anti-angiogenic effects. However, these effects, especially for flavonoids, have often been studied at un-physiologically high concentrations in both *in vitro* and *in vivo* experiments and their structure activity relationship has remained poorly defined for their biological effects at physiologically concentrations (Middleton, Kandaswami et al. 2000; Havsteen 2002; Monasterio, Urdaci et al. 2004; Touil, Fellous et al. 2009; Lam, Alex et al. 2012).

Regarding the positive effects of flavonoids on preventing ischemic stroke and coronary heart disease, the pro-angiogenic effect of flavonoids are of interest in this thesis. The purpose of this work therefore, was to identify the pro-angiogenic activity of the two most anti-angiogenic and bioactive flavonoids, genistein and naringenin, at concentrations that encompass those found in human plasma (5×10^{-6} M to 5×10^{-12} M) and to study their cellular mode of action in angiogenesis in addition to determining some structural motifs of flavonoids required for their pro-angiogenic activity. An *in vitro* rat aorta pro-angiogenesis assay was employed to screen for the pro- and anti-angiogenic activity of the test compounds. Although *in vitro* angiogenesis assays can be carried out rapidly and can provide initial valuable information, a large number of assays are required to counter the considerable degree of variability inherent in biological assays.

Initially, a series of eighteen flavonoids were screened with the *in vitro* rat aorta pro-angiogenesis bioassay at concentrations of 5×10^{-7} and 5×10^{-8} M which, as discussed earlier, are more likely to represent the physiological plasma concentrations derived from a normal diet.

The structural elements of the flavonoids which seem to be critical for their pro-angiogenic activity include the C4-oxo (C4=O) functional group conjugated with the C2-C3 double bond (Fotsis, Pepper et al. 1997; Schindler and Mentlein 2006) as found, for example, in the flavones (e.g., apigenin), isoflavones (e.g., genistein) and flavonols (e.g., quercetin) (Table 1.1 and 5.2). Interestingly, genistein with a C2-C3 double bond and naringenin without this bond both enhanced the angiogenesis activity at 5×10^{-7} M and 5×10^{-8} M (Fig. 5.2). To our knowledge this is the first time that these flavonoids have been tested at physiological concentrations (μM range and under) in a pro-angiogenesis assay.

The anti-angiogenic activity of genistein (1-250 μM) and the anti-tumour effect of naringenin (10,100 and 200 μM) has been reviewed for concentrations higher than 1 μM (Fotsis, Pepper et al. 1995; Manthey and Guthrie 2002; Buchler, Reber et al. 2004; Sasamura, Takahashi et al. 2004; Zhang, Du et al. 2009). Therefore, genistein, naringenin and five other flavonoids were selected for testing with the *in vitro* pro-angiogenesis assay at 5×10^{-6} M (Fig. 5.5). Interestingly none of the tested flavonoids showed significant pro-angiogenic activity. The notable point was that genistein showed anti-angiogenic activity at 5×10^{-6} M, and most interestingly enhanced angiogenic activity at 5×10^{-7} M and 5×10^{-8} M, which shows dose dependent activity for this flavonoid.

Because of the difference in the angiogenic activity of genistein, at 5×10^{-6} M compared to 5×10^{-7} M and 5×10^{-8} M, and to examine whether genistein can inhibit angiogenesis at high μM concentrations, as reported in many other studies (Fotsis, Pepper et al. 1993; Fotsis, Pepper et al. 1995; Buchler, Reber et al. 2004; Banerjee, Li et al. 2008), it was tested in the *in vitro* anti-angiogenesis rat aorta assay at 1×10^{-4} M and 2×10^{-4} M and shown to have a very significant dose dependent angiogenesis inhibitory effect which confirmed the observations reported previously (Fig. 5.6).

Seven selected pro-angiogenic flavonoids were also further tested for pro-angiogenic effects at 5×10^{-7} M and 5×10^{-8} M, with or without additional bFGF (12.5 ng/mL), to assess the effect of flavonoids on bFGF-stimulated angiogenesis. The results showed that adding extra bFGF did not have any positive effect on pro-angiogenic activity and the tested compounds showed more significant activity in the absences of extra bFGF

demonstrations that the 5% HIFCS used in the rat aorta pro-angiogenesis assay has enough bFGF to support the activity of test compounds (Fig. 5.3 and 5.4).

The C3-OH and C2-C3 double bond (flavanol) structural features, which were reported to have a positive effect on the anti-angiogenic activity (Fotsis, Pepper et al. 1997), were confirmed in our tests since quercetin and kaempferol showed no significant pro-angiogenic activity but the activity reached significant levels in the absence of the C2-C3 double bond in dihydroquercetin and dihydrokaempferol. Another example of this was found with apigenin and naringenin, with naringenin lacking the C2-C3 double bond and exhibiting significantly enhanced pro-angiogenic activity compared to apigenin (Schindler and Mentlein 2006). Also, when comparing the activity of isoflavone and 7-methoxy isoflavone, a positive effect of C7-OCH_3 on anti-angiogenic activity was demonstrated (Lam, Alex et al. 2012).

In our tests, generally acacetin with C7-OH, C5-OH and a methoxy group at C4′ (C4′-OCH_3) showed more pro-angiogenic activity (only at 5×10^{-7}M) compared to apigenin with C7-OH, C5-OH and C4′-OH (Fig. 5.2). These data suggest that C7-OH may enhance angiogenesis when it is accompanied by a methoxy group at C4′ on the flavonoid B ring. Moreover, the angiogenic effect of OH groups at C3′ and C4′ was investigated by comparing the pro-angiogenic activity of dihydroquercetin (C3′-OH and C4′-OH) with dihydrokaempferol (C4′-OH). The positive effect of (C3′-OH and C4′-OH) on the anti-angiogenic activity was confirmed when dihydroquercetin showed less pro-angiogenic activity compared to dihydrokaempferol (Fotsis, Pepper et al. 1997).

In our experiments, glycosylation at C7 had no significant effect on the pro-angiogenic activity of naringenin and naringenin-7-*O*-glucoside, consistent with the results of Lam that glycosylation does not affect the anti-angiogenic activity of high concentrations of flavonoids (Lam, Alex et al. 2012). 7-hydroxycoumarin with two phenolic rings and 2, 5-dihydroxy-1,4-benzoquinon with one phenolic ring were tested as a model for A and C, and C phenolic rings in a flavonoid structure, respectively. The significant pro-angiogenic activity of these two compounds shows the importance of both A and C phenolic rings in pro-angiogenic activity; although, 7-hydroxycoumarin showed slightly greater pro-angiogenic activity.

As angiogenesis is a complex and highly regulated process, several *in vitro* assays modelling major steps in this process have been employed to investigate the mode of action of naringenin and genistein and five other pro-angiogenic flavonoids (dihydro quercetin, dihydro kaempferol, 7-hydroxy coumarin, naringenin-7-*O*-glucoside, flavanone). The steps involved in angiogenesis, including cell proliferation, migration and tube formation, can be studied *in vitro* and these steps represent possible intervention targets for these angiogenesis modifying compounds (Auerbach, Lewis et al. 2003).

Initially, cell proliferation assays were conducted using serum-starved HUVEC in the presence and absence of bFGF (12.5 ng/mL). The rationale behind using bFGF, a potent mitogenic and pro-angiogenic growth factor, was to explore whether the test compounds can modify the mitogenic activity of bFGF. Sub-optimal concentrations of bFGF (12.5 ng/mL) were used (as in Chapter 4) to permit the enhancing or inhibiting effects of test compounds to be measured. Naringenin showed significant elevation in endothelial cell proliferation without bFGF and when adding bFGF, naringenin, 7-hydroxycoumarin and flavanone showed significant enhancement in cell proliferation at 5×10^{-7} M or 5×10^{-8} M (Fig. 5.9). These results are comparable with a study performed by Comalada, suggesting the absence of the C2-C3 double bond reduced the anti-proliferative effect of flavonoids used at high concentrations (Comalada, Ballester et al. 2006).

In separate experiments using naringenin at concentrations from 5×10^{-6} M to 5×10^{-12} M, significant mitogenic effects at concentrations from 5×10^{-6} M to 5×10^{-9} M, in the absence or presence of bFGF were observed which suggests a strong mitogenic effect of naringenin on endothelial cells (Fig. 5.10). In contrast, genistein in a similar test significantly inhibited HUVEC cell proliferation in the presence and absence of bFGF at 5×10^{-6} M and showed no significant enhancement of proliferation at 5×10^{-7} M and 5×10^{-8} M (Fig. 5.11). Also, in the presence of bFGF, the proliferative activity of genistein was less than the control for all tested concentrations which confirms the previous results showing genistein inhibits bFGF stimulated proliferation by inhibiting bFGF activity through attenuation of the activity of tyrosine kinases (Fotsis, Pepper et al. 1993; Fotsis, Pepper et al. 1995). Significantly enhanced proliferation induced by naringenin is comparable with the enhancement of angiogenesis by naringenin at almost

all concentrations tested and the anti-proliferative activity of genistein supports the results of inhibition of the overall process of angiogenesis by genistein at 5×10^{-6} M.

However, additional studies revealed that naringenin can significantly inhibit 3T3 mouse fibroblast proliferation (non-EC) at 5×10^{-6} M and 5×10^{-7} M in a dose dependent manner (Fig. 5.25A). It seems, therefore, that the effects of naringenin on cell proliferation are cell type-specific. Also, genistein showed approached statistically significant inhibition of 3T3 mouse fibroblast proliferation at 5×10^{-6} M (Fig. 5.25B). These results are compatible with studies claiming naringenin and genistein as anti-proliferative agents on cancer cell lines (Fotsis, Pepper et al. 1995; Shao, Alpaugh et al. 1998; Elattar and Virji 2000; Manthey and Guthrie 2002; Banerjee, Li et al. 2008; Zhang, Du et al. 2009; Qi, Weber et al. 2011).

In our study, none of the tested flavonoids showed significant enhancement or inhibition of endothelial cell migration/wound healing responses on a wounded confluent HMEC monolayer. Also, naringenin and genistein did not affect HMEC cell adhesion to the ECM components fibronectin and vitronectin. Interestingly, genistein could inhibit HUVEC tube formation at 5×10^{-6} M while naringenin could enhance this activity at 5×10^{-7} M.

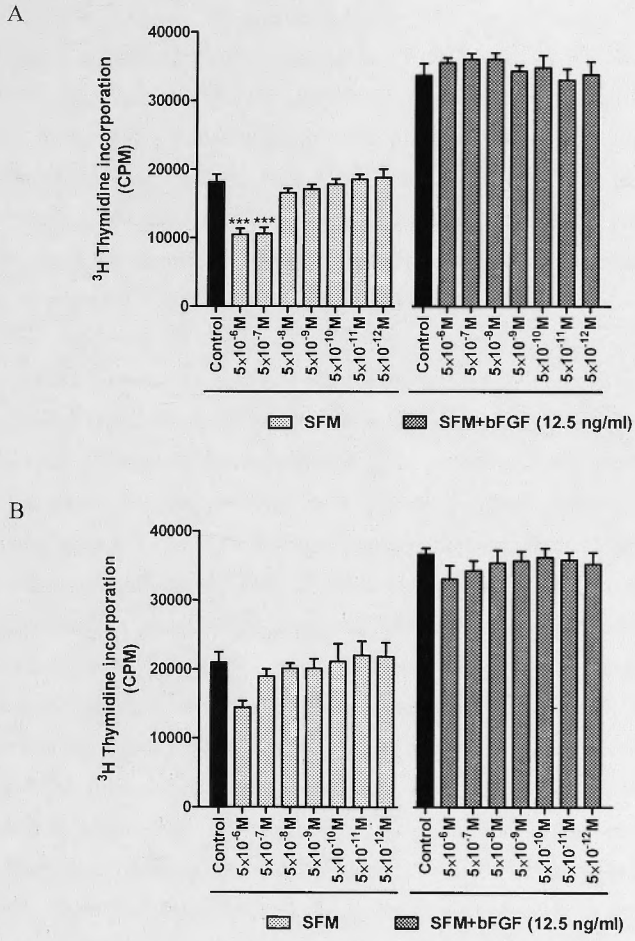


Figure 5.25. Effect of naringenin and genistein on 3T3 mouse fibroblast proliferation assay.

Effect of naringenin (A) and genistein (B) at concentrations from 5×10^{-6} M to 5×10^{-12} M on confluent serum-starved 3T3 mouse fibroblast proliferation in serum free media (SFM) with and without the mitogen bFGF (12.5 ng/mL). Control cultures contained the same diluent dilution as the test compounds. Cell proliferation was measured as ^{3}H thymidine incorporation after 24 hours incubation. Data analysis was performed by Student–Newman–Keuls test after one-way ANOVA comparing each group to control in each treatment. Error bars represent SEM (n=6). ***, ≤ 0.001. A two-way ANOVA data analysis showed significant difference for all tested concentrations comparing their effect in SFM to SFM+bFGF (12.5 ng/mL) treatments, $P \leq 0.0001$.

In previous studies, the ability of genistein to inhibit bFGF induced endothelial cell migration was reported by Fotsis; however, the concentrations used were un-physiological at 100 μM (Fotsis, Pepper et al. 1995). Genistein, has also been reported to induce cell-integrin (fibronectin) interactions and to decrease the rate of metastasis in prostate cancer 2 hours after adding 20-50 μM and 3 days after adding 1 μM of genistein, respectively (Bergan, Kyle et al. 1996; Lakshman, Xu et al. 2008; Pavese, Farmer et al. 2010). Inhibition of endothelial cell tube formation by genistein also has been reported but again this was at an un-physiological concentration of 150 μM (Fotsis, Pepper et al. 1993; Fotsis, Pepper et al. 1995).

Naringenin has been reported in a number of studies to inhibit cell proliferation in a variety of cell types, but the effect of naringenin on cell migration and tube formation has not been explored in depth previousely. It has been shown that naringenin can inhibit the migration of *Dictyostelium* cells (a model for identifying cellular processes in health and disease including cell migration (Egelhoff and Spudich 1991) at 50 μM (Russ, Martinez et al. 2006). Oral administration of naringenin (100 mg/kg) in mice with breast cancer significantly decreased the number of lung metastatic colonies compared to control groups (Qin, Jin et al. 2011). However, naringenin had no effect on colon epithelial cell migration in a non-tumourigenic cell line at 1, 50 and 300 μM (Fenton and Hord 2004). There has been little work done on the effect of naringenin on integrin-ECM interactions but naringenin has been shown to possess anti-fibrotic properties by decreasing ECM expression in rat hepatic stellate cells and fibrotic progression at 50 μM (Liu, Wang et al. 2006). Another study showed that naringenin can inhibit ECM production by nasal polyp-derived fibroblasts at 40 μM (Jung, Park et al. 2012) and it has also been shown that oral administration of 10 μM of naringenin (plasma levels 2-6 μM) for 21 days can significantly decrease the metastatic frequencies of B16-F10 melanoma cells by enhancing cell-cell and cell-ECM adhesion (Lentini, Forni et al. 2007).

In conclusion, the results obtained with an *in vitro* rat aorta pro-angiogenesis assay and 18 flavonoids demonstrated that some of the flavonoids exhibited either pro- or anti-angiogenic activity at physiological concentrations (5×10^{-7} M and 5×10^{-8} M). We also confirmed the critical effect of the C2-C3 double bond conjugated with C4=O on anti-angiogenic activity. Also, positive effects of C3′-OH and C4′-OH, C7-OCH_3 and C3-OH on anti-angiogenic activity were demonstrated, especially when these

structural motifs are combined with the C2-C3 double bond. The isoflavonoid, genistein, inhibited *in vitro* angiogenesis at 5 μM and exhibited pro-angiogenic activity at sub-μM concentrations (5×10^{-7} M and 5×10^{-8} M). The anti-angiogenic effect of genistein at 5×10^{-6} M was supported by the observed anti-proliferative and inhibition of tube formation activities. In contrast, the flavanone, naringenin, enhanced pro-angiogenic activity at 5×10^{-7} M to 5×10^{-11} M. This activity was supported by its strong mitogenic activity, with or without bFGF (12.5 ng/mL), on HUVEC and its ability to enhance HUVEC tube formation at 5×10^{-7} M.

The key conclusion from these findings is that some dietary flavonoids may exhibit pro-angiogenic activity at their physiological plasma concentrations. Thus, a normal diet containing a moderate level of vegetables and fruit may result in therapeutic angiogenesis which could help ischemic conditions such as stroke and heart disease.

Chapter 6

Final Discussion and Future Directions

6.1. Final discussion

Plant natural products and their derivatives continue to be invaluable sources of therapeutic agents to enhance human health and to treat disease (Koehn and Carter 2005). This thesis has identified new potential therapeutics that regulate angiogenesis. The process of angiogenesis involves the formation of new blood vessels from pre-existing blood vessels. Angiogenesis is a tightly controlled process and either excessive or insufficient angiogenesis has been associated with many diseases. For example, excessive angiogenesis contributes to tumour progression, psoriasis, blindness induced by age-related macular degeneration (AMD) and arthritis (Folkman 2000; Carmeliet 2003; Folkman 2007). On the other hand, insufficient angiogenesis leads to physiological conditions including hypertension, impaired wound healing (Tonnesen, Feng et al. 2000; Bao, Kodra et al. 2009) and various ischemic conditions such as cardiovascular disease (Pandya, Dhalla et al. 2006) and stroke (Beck and Plate 2009; Font, Arboix et al. 2010).

In 1971 Folkman introduced the concept of "anti-angiogenesis" as a therapeutic strategy and since then a diverse and extensive range of anti-angiogenic molecules have been isolated, identified and tested in clinical trials (Pezzuto 1997; Newman 2011). However, pro-angiogenic drugs are less well researched and only a limited number have been studied in clinical settings. Therefore, the aim of this research was to screen for natural products with pro-angiogenic activity using an activity-guided bioassay strategy to purify and isolate sufficient material to enable their identification and to study their cellular mode of action using human cell lines in a series of *in vitro* angiogenesis assays.

There is a substantial body of correlative studies associating the consumption soybeans (*Glycine max)* with a number of health benefits (Barnes 1998; Tham, Gardner et al. 1998). Soybeans are a rich source of flavonoids with anti-oxidant and phytoestrogen properties and preliminary studies in the Djordjevic and Parish laboratories had indicated that they may also be a source of compounds with the ability to either enhance or inhibit angiogenesis to different extents (Du Fall 2009). Therefore, the work described in this thesis was directed at isolating and identifying these active components from soybean xylem sap and studying their cellular mode of action. It was also decided to examine the pro-angiogenic activity and structure-activity relationship

of a selection of flavonoids, including naringenin and genistein, that also occur in soybeans but to test these at concentrations likely to be achieved in plasma following dietary consumption.

6.2. Major research findings of this thesis

6.2.1. Screening, fractionation and structural elucidation of compounds from soybean xylem sap that promote angiogenesis

Soybeans were cultured under several regimes and the relative abundance of substances in the fractionated xylem sap material was found to be quite varied. As a subtropical plant, ideal growth conditions in glasshouses were limited to the warmer months with a long day length. The optimisation of the yield of pro-angiogenic materials was not systematically pursued but, generally, the more robust the plants grew, the higher the relative yield of xylem sap and pro-angiogenic activity. Plants fertilised with 10 mM KNO_3 produced a higher abundance of angiogenic-active metabolites than plants that relied on symbiotic nitrogen fixation as the major source of organic nitrogen (Fig. 3.3A and 3.3B). The successive use of 10 kDa and 3 kDa molecular weight spin filters and HPLC in combination with an *in vitro* rat aorta bioassay was used to size fractionate the material and guide the collection of three fractions (***FK1***, ***FK2*** and ***P6***) from xylem sap (Fig. 3.3). However, ***P6*** appeared to be of low and variable abundance and poor stability. In contrast, approximately 400 µg of ***FK1*** and ***FK2*** was obtained following semi-preparative HPLC (C18) (Fig. 3.7). Structural elucidation using NMR (^{1}H and ^{13}C) and high resolution accurate mass LC/MS, showed the pro-angiogenic compounds to be *erythro*-guaiacylglycerol-8-*O*-4'-coniferyl alcohol (***FK1***), and *threo*-guaiacylglycerol-8-*O*-4'-coniferyl alcohol (***FK2***) (Fig. 3.10 and 3.11and Table 3.2 and 3.3).

FK1 and ***FK2*** are both lignans and are derived from the free radical dimerisation of monolignols which are the precursors of the structural element lignin in plant secondary cell walls. Lignans are formed in plants firstly by the production of glycosylated monolignols in the cytoplasm from phenylalanine and these molecules are then transported through the cell membrane to the apoplast. Polymerisation is thought to begin after the removal of the glycosyl group (Boerjan, Ralph et al. 2003). The lignans isolated in this thesis are dimeric derivatives that apparently accumulate to appreciable

levels in the xylem sap before being polymerised to lignin. These compounds would therefore be expected to occur to some extent in the vascular tissue of soybeans and be present in leaves, stems and possibly roots. It is not known to what extent these compounds would occur in the edible part of soybeans (the bean).

It is interesting to note that both ***FK1*** and ***FK2*** conform to the physico-chemical parameters outlined by Lipinski (1997) in his Rule of Five for desirable oral drug properties. ***FK1*** and ***FK2*** possessed a HBD = 4 ($\leq$ 5), HBA = 7 ($\leq$ 10), M_r = 376 ($\leq$ 500 Da), logP = 1.58 ($\leq$ 5). These values were derived using the online calculator available at http://www.vlsd.com/JME_EditorOK.dir/run_jlogp.html, accessed 2nd April 2013. The physico-chemical parameters of ***FK1*** and ***FK2*** are also in conformity with some of the more stringent requirements that have since been developed (see section 1.3 in Chapter 1), with logP = 1.58 ($\leq$ 3), PSA = 108.6 ($\leq$ 140 Å^2) (values obtained using the online calculator available from http://www.molinspiration.com/cgi-bin/properties, accessed 2nd April 2013). Although the number of rotatable bonds at 9 is clearly higher than desirable ($\leq$ 3), this is the only parameter which shows a less than ideal value.

6.2.2. Determination of the cellular mode of action of *FK1* and *FK2* using human cell lines

In chapter 4, following the identification and structural determination of ***FK1*** and ***FK2*** from soybean, synthetic ***FK1*** and an independently derived natural source of ***FK2*** (isolated from *Bretschneidera sinensis*) were obtained firstly to confirm their pro-angiogenic activity and secondly, to study the cellular mechanism of their ability to modulate angiogenesis. The *in vitro* pro-angiogenesis assay (testing concentrations in the range of 5×10^{-6} M to 5×10^{-9} M) confirmed that the newly obtained ***FK1*** and ***FK2*** possess essentially identical pro-angiogenic activity to the soybean-derived compounds (Fig. 4.2). Interestingly, the pro-angiogenic activity increased from 5×10^{-6} M to 5×10^{-8} M which might suggest that using higher concentrations (i.e., > μM) may not increase their pro-angiogenic activity as ANOVA analysis of ***FK2*** cell proliferation showed a significant difference between 5×10^{-6} M to 5×10^{-8} M and 5×10^{-9} M in the presence of bFGF. Moreover, as mentioned in chapter 4, both, ***FK1*** and ***FK2*** have been previously found to have cytotoxic and chemo-preventative effects on human colon carcinoma, human hepatocellular carcinoma and prostate cancer in the high μM range (80 to 150 μM) of concentrations (Lee, Song et al. 2007; Han, Wang et al. 2008).

Indeed, higher concentrations of ***FK1*** and ***FK2*** may prove inhibitory or toxic, so it is important to determine the therapeutic range of these molecules.

The cellular mode of action of synthetic ***FK1*** and naturally derived ***FK2***, was investigated using several *in vitro* assays modelling the major steps in angiogenesis, including endothelial cell proliferation, migration, tube formation and adhesion to ECM components (fibronectin and vitronectin) (section 4.4.1 to 4.4.5). It was found that these compounds can significantly enhance HUVEC endothelial cell proliferation (mainly in the presence of bFGF) and HUVEC and HMEC tube formation on an artificial ECM, Matrigel. However, they did not affect HMEC migration and adhesion to fibronectin and vitronectin. Therefore, the proposed mechanism of action for pro-angiogenic activity of these compounds is the possible potentiation of the potent mitogen, bFGF and its downstream signalling.

The synthetic ***FK1*** was also tested in an anti-malaria drug bioassay but no inhibitory effect on *Plasmodium* viability at concentrations from 10^{-4} M to 10^{-14} M was found compared to the well-known anti-malaria drug, artemisinin. This confirms the study by Ma et al. (2006) who showed that an HPLC fraction containing both ***FK1*** and ***FK2*** from *Grewia bilamellata* had no anti-malaria effect against *P.faciparum* (Ma, Zhang et al. 2006).

6.2.3. Investigate the pro-angiogenic activity and structure-activity relationship, at physiological concentrations, of a selection of flavonoids, including naringenin and genistein

Flavonoids have been studied for a variety of beneficial biological activities in humans including prevention of cardiovascular disease and anti-angiogenic activity (Ren, Qiao et al. 2003). However, these effects have often been studied at high, non-physiological, concentrations (high μM range) in both *in vitro* and *in vivo* experiments. Therefore, their structure-activity relationship has remained poorly defined at physiological concentrations ($\leq$ 1 μM) which would result from a diet containing normal levels of fruit and vegetables (Middleton, Kandaswami et al. 2000; Monasterio, Urdaci et al. 2004; Touil, Fellous et al. 2009; Lam, Alex et al. 2012).

In chapter 5, 18 flavonoids (Table 5.2) were screened with an *in vitro* pro-angiogenic bioassay at physiological concentrations ranging from 5×10^{-7} M to 5×10^{-8} M and seven flavonoids (naringenin, genistein, naringenin-7-*O*-glucoside, dihydrokaempferol, dihydroquercetin, 7-hydroxycoumarin and flavanone) showed consistent pro-angiogenic activity. A key finding of this study was that none of the tested flavonoids were pro-angiogenic at 5×10^{-6} M. Moreover, the isoflavonoid, genistein, inhibited *in vitro* angiogenesis at 5 μM and enhanced pro-angiogenic activity at sub-μM concentrations (5×10^{-7} M and 5×10^{-8} M). The angiogenesis inhibitory effect of genistein at 5×10^{-6} M was supported by inhibition of 3T3 mouse fibroblast proliferation (Fig. 5.25B) as well as HUVEC proliferation and HUVEC tube formation activities and confirmed the results found by Akiyama et al., 1987 and Fotsis et al., 1993 showing that genistein inhibits bFGF- and VEGF-driven endothelial cell proliferation, migration, and tube formation (Akiyama, Ishida et al. 1987; Fotsis, Pepper et al. 1993). Naringenin significantly promoted angiogenesis at concentrations of 5×10^{-9} M to 5×10^{-10} M (Fig. 5.8). The results obtained from its strong mitogenic activity on HUVEC proliferation and HUVEC tube formation (Fig. 5.10), provided the evidence for this flavanone's pro-angiogenic activity. However, naringenin could significantly inhibit 3T3 mouse fibroblast proliferation at 5×10^{-6} M and 5×10^{-7} M (Fig. 5.25A).

The pro-angiogenic response of a series of 18 flavonoids revealed some important structural motifs that were important for activity at physiological concentrations (5×10^{-7} M and 5×10^{-8} M). The results confirmed that, for anti-angiogenic activity, the critical effect of the C2-C3 double bond being conjugated with C4=O. Also, flavonoids with C3′-OH and C4′-OH, C7-OCH_3 and C3-OH with the C2-C3 double bond showed anti-angiogenic activity. All flavonoids with no C2-C3 double bond showed significant pro-angiogenic activity, including hesperetin and the seven flavonoids mentioned above.

6.3. Future directions

As described in chapter 1, angiogenesis is a complex and highly regulated process. The studies performed in this thesis identified two groups of pro-angiogenic molecules, ***FK1*** and ***FK2*** (neolignans), and genistein and naringenin (flavonoids), each group appearing to have novel mechanisms of action. In order to investigate their mode of action in more details and also discover the other structural related molecules that possess pro-angiogenic activity, further experiments are suggested.

6.3.1. *In vivo* angiogenesis bioassay

In this thesis an *in vitro* rat aorta angiogenesis bioassay was employed to screen for compounds with pro-angiogenic activity. As mentioned in chapter 1 (section 1.10), *in vitro* angiogenesis assays are the most valuable first step in identifying compounds that modulate angiogenesis. However, *in vivo* assays provide a more comprehensive insight as to how the test compounds might affect the angiogenic process. Therefore, in order to validate the *in vitro* angiogenesis results obtained from ***FK1*** and ***FK2***, and also genistein and naringenin at sub-μM concentrations, *in vivo* angiogenesis assays in animal models such as chick chorioallantoic membrane (CAM) and Matrigel plug assays could be used (Auerbach, Lewis et al. 2003). Recently, biologically-active Nod factor and Nod factor-like compounds were tested in an *in vivo* Matrigel plug assay in collaboration with Professor Levon Khachigian, at UNSW. In this assay, a series of individual Matrigel plugs containing different Nod factors were assayed against a bFGF containing control to confirm the *in vitro* pro-anti-angiogenic activity of the test compounds (Djordjevic, Bezos et al. 2013). It would be interesting, therefore, to extend our study to look at the *in vivo* angiogenic activity of ***FK1*** and ***FK2***.

6.3.2. Studies on *FK2*, and *FK1* and *FK2* analogues

As mentioned in chapter 4, following structural determination of soybean xylem-sap-derived ***FK1*** and ***FK2***, ***FK1*** was obtained from BOC Sciences, NY, USA and naturally derived ***FK2*** (from *Bretschneidera sinensis*) was provided as a gift from Prof. Dr. Wei-Dong Zhang and Dr. Shan Lei from the Second Military Medical University, Shanghai, China. Although, the observed angiogenic activity of ***FK2*** was higher than ***FK1*** (Fig. 4.2 and 4.3), the quantity of ***FK2*** provided was not sufficient to complete the cellular mode of action studies. In theory, the 8-*O*-4′ coupling of coniferyl alcohol could result in forming a total of four stereo isomers, the *erythro*-7S, 8S/7R, 8R and *threo*-7R, 8S/7S, 8R. Based on the greater effects observed with ***FK2*** it would be of interest to complete the cellular mode of action studies of ***FK2*** and also to examine the individual stereoisomers for pro-angiogenic activity (Fig. 3.9).

Indeed it would be interesting to test other members of the large lignin family of compounds (Fig. 3.13), particularly the lignans (C8-C8′ dimers) which are the most abundant dimers in dietary food (Fig. 4.17) (Umezawa 2007) and which have been

shown to be converted to mammalian lignans after digestion by the microflora in the human large intestine (Fig. 4.18) (Heinonen, Nurmi et al. 2001). These molecules have already demonstrated a wide range of bioactivity in humans, including anti-breast, colon and prostate cancer, oestrogenic and anti-oestrogenic activities, anti-inflammatory activity and positive effects on cardiovascular disease (Table 4.2). Therefore, the general chemical family of lignans and neolignans are also good candidates to be investigated for angiogenesis modulating activity.

6.3.4. Other active xylem sap fractions

Previously, it was shown that xylem sap contains both anti- and pro-angiogenic fractions (Du Fall 2009). This was followed up in this thesis by identifying the late eluting pro-angiogenic fractions (Fig. 3.1, 3.2 and 3.3). The late eluting fractions (i.e., the ones containing ***FK1***, ***FK2*** and ***P6***) were examined because they were relatively less complex chemically. The ***P6*** pro-angiogenic fraction was only partially characterised due to its low abundance and unstable nature. Several other fractions eluting at earlier times in the HPLC fractionation were not examined further because (a) they appeared to co-elute with other materials and, therefore, would be more difficult to purify and (b) they were more hydrophilic and hence less desirable from a potential therapeutic point of view. Therefore the active components in these fractions remain unidentified. It is possible that they may represent more hydrophilic derivatives of ***FK1***, ***FK2*** and/or ***P6***, but this remains to be determined. Nevertheless, these active fractions and ***P6*** may be worthy of further exploration.

6.3.5. Molecular mode of action studies

6.3.5.1. Investigate the possible molecular targets and mechanisms affected by *FK1/FK2*

Having established that ***FK1*** and ***FK2*** can significantly enhance endothelial cell proliferation (mainly in the presence of bFGF) and tube formation on an artificial ECM, it would be valuable to elucidate their molecular mode of action and to investigate the pro-angiogenesis phenomenon at the genetic and regulatory level. It is possible that ***FK1*** and ***FK2*** potentiate the potent mitogen, bFGF and its downstream signalling and most possibly affect MAPK/PKC (mitogenic activity) in cell proliferation and the

laminin-integrin ($\alpha 6\beta_1$) interaction in tube formation. Also, ***FK1*** and ***FK2*** might interfere with the bFGF mechanism of regulating angiogenesis by inducing MMP-2 and MMP-9 to degrade the ECM in the formation of sprouts or enhancing cell-cell adhesion via cadherins in tube formation (Fig. 6.2). In order to investigate the possible pathways affected by ***FK1***/***FK2*** treatment, differential gene expression analyses (e.g., real-time RT-PCR, microarray or RNA-Seq technologies) could be used to determine the mRNA content of the endothelial cells and has been used to diagnose diseases such as cancer (Maher, Kumar-Sinha et al. 2009). They can provide information on gene alleles, differently spliced transcripts, post-transcriptional mutations or modifications and gene fusions (Maher, Kumar-Sinha et al. 2009).

Furthermore, at the protein level, an analysis of post-translational modifications (e.g., phosphorylation analysis by mass spectrometry, photo affinity labelling and protein microarrays) can also be employed. Briefly, phosphorylation studies are able to identify which proteins are being activated by a certain treatment. Photo affinity labelling involves labelling the small molecule with either a tag which can be used to purify the protein to allow subsequent identification, or a photoreactive group (e.g., an azido moiety) that can be used to visualise the cellular location of the interacting protein. Protein microarrays consist of a chip which contains a support surface such as a glass slide to which specific proteins are bound. The reaction between the probe (small bioactive molecules typically labelled with fluorescent moiety) and the immobilised protein emits a fluorescent signal which can be read by a laser scanner.

Also, at the protein level, monoclonal antibodies (mAbs) tagged with a marker (e.g., enzyme in ELISA) could also be employed to follow differential expression of a target protein. These techniques would also allow the gene expression profiles induced by MAPK, PKC, and their intermediate molecules and also α6-integrin expression to be quantified and compared in endothelial cells cultured with and without ***FK1*** and ***FK2***.

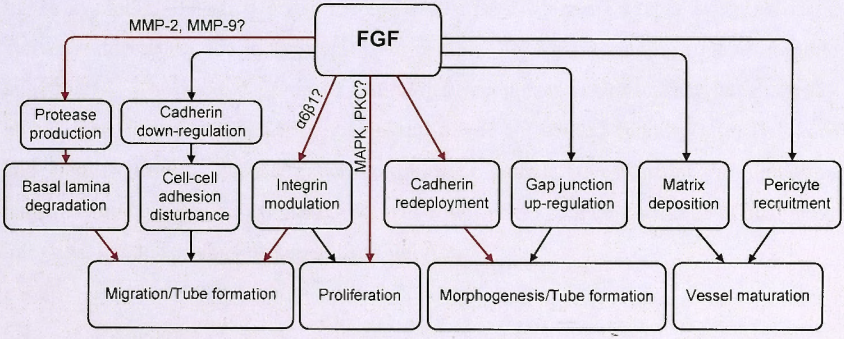


Figure 6.2. Possible *FK1*/*FK2* potentiation of the potent mitogen, bFGF, and its downstream signalling in endothelial cell.

In this thesis, it is suggested that ***FK1*/*FK2*** affect MAPK and PKC signalling pathways required for full mitogenic activity and the laminin-integrin ($\alpha6\beta_1$) interaction in tube formation. Also, they may induce MMP-2 and MMP-9 production/activation to degrade the ECM as well as enhancing cell-cell adhesion via cadherins during endothelial cell tube formation.

6.3.5.2. Investigate the effect of *FK1/FK2* on the affinity state of integrins

FK1 and ***FK2*** did not affect integrin-mediated ECM adhesion using fibronectin and vitronectin. However, this could be because integrins are already in a high affinity state in the cell lines used in the assays and, therefore, it would be difficult to assess the effect of pro-angiogenic compounds on inducing integrin affinity. This could be investigated by using cell lines in which the integrins are in an inactive state such as in freshly isolated HUVEC or resting lymphocytes. In order to investigate the effect of ***FK1/FK2*** on the conformational state of integrins, mAbs can be used to distinguish the active and inactive conformations of integrins. In this regard, there are two types of mAbs available. The first group recognises the ligand-binding site of integrins in the active conformation due to their ability to compete for ligand binding (ligand-mimetic antibodies). The second group of antibodies recognises integrins that have already bound their ligand (anti-CLIBS, cation-and-ligand influenced binding site) (Bazzoni and Hemler 1998). Further experiments might include investigating the signalling pathways being activated by ***FK1/FK2*** that could result in a conformational change in integrins, i.e., inside-outside signalling (Fig. 6.2).

6.4. Conclusion

This study has successfully identified and characterised two novel pro-angiogenic compounds, *erythro*-guaiacylglycerol-8-*O*-4'-coniferyl alcohol (***FK1***), and *threo*-guaiacylglycerol-8-*O*-4'-coniferyl alcohol (***FK2***) in soybean using an activity-guided fractionation approach. Both ***FK1*** and ***FK2*** could significantly enhance *in vitro* endothelial cell proliferation and tube formation on an artificial ECM, possibly by potentiating the potent mitogen, bFGF and its downstream signalling.

In addition, this thesis showed that two flavonoids (genistein and naringenin) that have been reported to be anti-angiogenic at high concentrations possess pro-angiogenic activity at lower (more physiologically relevant, sub-μM) concentrations using an *in vitro* rat aorta angiogenesis assay. These flavonoids and ***FK1*** and ***FK2*** have the potential to be developed for the therapeutic treatment of aberrant angiogenesis-related conditions such as cardiovascular disease, ischemia, stroke, chronic wounds and hypertension.

An intriguing question that arises from the work described in this thesis is; "Why should the neolignans be able to influence such a crucial process as angiogenesis?" It is possible that Toll-like receptors (TLRs) and other pattern recognition receptors (PRR) on the vascular endothelial cells that normally recognise pathogen-associated molecular patterns (PAMPs) are activated by bacterially derived molecules that are structurally similar to the neolignans. Clearly additional work is required to investigate this intriguing possibility.

References

Aehle, E., U. Muller, et al. (2011). "Lignans as food constituents with estrogen and antiestrogen activity." Phytochemistry **72**(18): 2396-2405.

Agarwal, R., C. Agarwal, et al. (2006). "Anticancer potential of silymarin: from bench to bed side." Anticancer Research **26**(6B): 4457-4498.

Aherne, S. A. and N. M. O'Brien (2002). "Dietary flavonols: Chemistry, food content, and metabolism." Nutrition **18**(1): 75-81.

Akiyama, T., J. Ishida, et al. (1987). "Genistein, a Specific Inhibitor of Tyrosine-Specific Protein-Kinases." Journal of Biological Chemistry **262**(12): 5592-5595.

Alcaraz, M. J. and M. L. Ferrandiz (1987). "Modification of Arachidonic Metabolism by Flavonoids." Journal of Ethnopharmacology **21**(3): 209-229.

Alitalo, K. (2011). "The lymphatic vasculature in disease." Nature Medicine **17**(11): 1371-1380.

Aranda, E. and G. I. Owen (2009). "A semi-quantitative assay to screen for angiogenic compounds and compounds with angiogenic potential using the EA.hy926 endothelial cell line." Biological Research **42**(3): 377-389.

Arnaoutova, I. and H. K. Kleinman (2010). "In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract." Nature Protocols **5**(4): 628-635.

Auerbach, R., R. Lewis, et al. (2003). "Angiogenesis assays: A critical overview." Clinical Chemistry **49**(1): 32-40.

Aviles, R. J., B. H. Annex, et al. (2003). "Testing clinical therapeutic angiogenesis using basic fibroblast growth factor (FGF-2)." British Journal of Pharmacology **140**(4): 637-646.

Bagli, E., M. Stefaniotou, et al. (2004). "Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3'-kinase activity." Cancer Research **64**(21): 7936-7946.

Balunas, M. J. and A. D. Kinghorn (2005). "Drug discovery from medicinal plants." Life Sciences **78**(5): 431-441.

Banerjee, S., Y. W. Li, et al. (2008). "Multi-targeted therapy of cancer by genistein." Cancer Letters **269**(2): 226-242.

Bao, P., A. Kodra, et al. (2009). "The Role of Vascular Endothelial Growth Factor in Wound Healing." Journal of Surgical Research **153**(2): 347-358.

Barclay, C., A. W. Li, et al. (2005). "Basic fibroblast growth factor (FGF-2) overexpression is a risk factor for esophageal cancer recurrence and reduced survival, which is ameliorated by coexpression of the FGF-2 antisense gene." Clinical Cancer Research **11**(21): 7683-7691.

Barczyk, M., S. Carracedo, et al. (2010). "Integrins." Cell and Tissue Research **339**(1): 269-280.

Barnes, S. (1998). "Evolution of the health benefits of soy isoflavones." Proceedings of the Society for Experimental Biology and Medicine **217**(3): 386-392.

Bates, D. O., N. J. Hillman, et al. (2002). "Regulation of microvascular permeability by vascular endothelial growth factors." Journal of Anatomy **200**(6): 581-597.

Battle, G. M., G. O. Kyd, et al. (2012). "Up the Garden Path: A Chemical Trail through the Cambridge University Botanic Garden." Journal of Chemical Education **89**(11): 1390-1394.

Baucher, M., B. Monties, et al. (1998). "Biosynthesis and genetic engineering of lignin." Critical Reviews in Plant Sciences **17**(2): 125-197.

Bauer, J., M. Margolis, et al. (1992). "Invitro Model of Angiogenesis Using a Human Endothelium-Derived Permanent Cell-Line - Contributions of Induced Gene-Expression, G-Proteins, and Integrins." Journal of Cellular Physiology **153**(3): 437-449.

Baumgartner, L., S. Sosa, et al. (2011). "Lignan Derivatives from Krameria lappacea Roots Inhibit Acute Inflammation in Vivo and Pro-inflammatory Mediators in Vitro." Journal of Natural Products **74**(8): 1779-1786.

Bazzoni, G. and M. E. Hemler (1998). "Are changes in integrin affinity and conformation overemphasized?" Trends in Biochemical Sciences **23**(1): 30-34.

Beck, H. and K. H. Plate (2009). "Angiogenesis after cerebral ischemia." Acta Neuropathologica **117**(5): 481-496.

Beecher, G. R. (2003). "Overview of dietary flavonoids: Nomenclature, occurrence and intake." Journal of Nutrition **133**(10): 3248s-3254s.

Begum, A. N., C. Nicolle, et al. (2004). "Dietary lignins are precursors of mammalian lignans in rats." Journal of Nutrition **134**(1): 120-127.

Benavente-Garcia, O. and J. Castillo (2008). "Update on uses and properties of Citrus flavonoids: New findings in anticancer, cardiovascular, and anti-inflammatory activity." Journal of Agricultural and Food Chemistry **56**(15): 6185-6205.

Benavente-Garcia, O., J. Castillo, et al. (1997). "Uses and properties of Citrus flavonoids." Journal of Agricultural and Food Chemistry **45**(12): 4505-4515.

Benderoth, M., S. Textor, et al. (2006). "Positive selection driving diversification in plant secondary metabolism." Proceedings of the National Academy of Sciences of the United States of America **103**(24): 9118-9123.

Bergan, R., E. Kyle, et al. (1996). "Genistein-stimulated adherence of prostate cancer cells is associated with the binding of focal adhesion kinase to beta-1-integrin." Clinical & Experimental Metastasis **14**(4): 389-398.

Bergers, G. and D. Hanahan (2008). "Modes of resistance to anti-angiogenic therapy." Nature Reviews Cancer **8**(8): 592-603.

Beutler, J. A., E. Hamel, et al. (1998). "Structure-activity requirements for flavone cytotoxicity and binding to tubulin." Journal of Medicinal Chemistry **41**(13): 2333-2338.

Bickerton, G. R., G. V. Paolini, et al. (2012). "Quantifying the chemical beauty of drugs." Nature Chemistry **4**(2): 90-98.

Birt, D. F., R. S. Markin, et al. (1998). "Dietary lignin, an insoluble fiber, enhanced uterine cancer but did not influence mammary cancer induced by N-methyl-N-nitrosourea in rats." Nutrition and Cancer-an International Journal **31**(1): 24-30.

Bloedon, L. T., S. Balikai, et al. (2008). "Flaxseed and cardiovascular risk factors: Results from a double blind, randomized, controlled clinical trial." Journal of the American College of Nutrition **27**(1): 65-74.

Bloedon, L. T. and P. O. Szapary (2004). "Flaxseed and cardiovascular risk." Nutrition Reviews **62**(1): 18-27.

Boerjan, W., J. Ralph, et al. (2003). "Lignin biosynthesis." Annual Review of Plant Biology **54**: 519-546.

Boilly, B., A. S. Vercoutter-Edouart, et al. (2000). "FGF signals for cell proliferation and migration through different pathways." Cytokine & Growth Factor Reviews **11**(4): 295-302.

Bootle-Wilbraham, C. A., S. Tazzyman, et al. (2000). "Fibrinogen E-fragment inhibits the migration and tubule formation of human dermal microvascular endothelial cells in vitro." Cancer Research **60**(17): 4719-4724.

Boue, S. M., T. E. Wiese, et al. (2003). "Evaluation of the estrogenic effects of legume extracts containing phytoestrogens." Journal of Agricultural and Food Chemistry **51**(8): 2193-2199.

Bouis, D., Y. Kusumanto, et al. (2006). "A review on pro- and anti-angiogenic factors as targets of clinical intervention." Pharmacological Research **53**(2): 89-103.

Brenk, R., A. Schipani, et al. (2008). "Lessons learnt from assembling screening libraries for drug discovery for neglected diseases." Chemmedchem **3**(3): 435-444.

Buchler, P., H. A. Reber, et al. (2004). "Antiangiogenic activity of genistein in pancreatic carcinoma cells is mediated by the inhibition of hypoxia-inducible factor-1 and the down-regulation of VEGF gene expression." Cancer **100**(1): 201-210.

Buck, K., A. K. Zaineddin, et al. (2011). "Estimated enterolignans, lignan-rich foods, and fibre in relation to survival after postmenopausal breast cancer." British Journal of Cancer **105**(8): 1151-1157.

Buer, C. S., N. Imin, et al. (2010). "Flavonoids: New Roles for Old Molecules." Journal of Integrative Plant Biology **52**(1): 98-111.

Bugianesi, R., G. Catasta, et al. (2002). "Naringenin from cooked tomato paste is bioavailable in men." Journal of Nutrition **132**(11): 3349-3352.

Bunzel, M., J. Ralph, et al. (2004). "Lignins and ferulate-coniferyl alcohol cross-coupling products in cereal grains." Journal of Agricultural and Food Chemistry **52**(21): 6496-6502.

Bunzel, M., J. Ralph, et al. (2001). "Diferulates as structural components in soluble and insoluble cereal dietary fibre." Journal of the Science of Food and Agriculture **81**(7): 653-660.

Burda, S. and W. Oleszek (2001). "Antioxidant and antiradical activities of flavonoids." Journal of Agricultural and Food Chemistry **49**(6): 2774-2779.

Butler, M. S. (2004). "The role of natural product chemistry in drug discovery." Journal of Natural Products **67**(12): 2141-2153.

Campbell, M. M. and R. R. Sederoff (1996). "Variation in lignin content and composition - Mechanism of control and implications for the genetic improvement of plants." Plant Physiology **110**(1): 3-13.

Cannell, R. J. P. (1998). Methods in Biotechnology 4. Natural Products Isolation. Totowa, Humana Press Inc.

Cao, G. H., E. Sofic, et al. (1997). "Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationships." Free Radical Biology and Medicine **22**(5): 749-760.

Cao, Y. H. and R. H. Cao (1999). "Angiogenesis inhibited by drinking tea." Nature **398**(6726): 381-381.

Carmeliet, P. (2003). "Angiogenesis in health and disease." Nature Medicine **9**(6): 653-660.

Carmeliet, P. (2005). "Angiogenesis in life, disease and medicine." Nature **438**(7070): 932-936.

Carmeliet, P. and R. K. Jain (2011). "Molecular mechanisms and clinical applications of angiogenesis." Nature **473**(7347): 298-307.

Casagrande, F. and J. M. Darbon (2001). "Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1." Biochemical Pharmacology **61**(10): 1205-1215.

Cassidy, A. (2006). "Factors affecting the bioavailability of soy isoflavones in humans." Journal of Aoac International **89**(4): 1182-1188.

Cassidy, A., B. Hanley, et al. (2000). "Isoflavones, lignans and stilbenes - origins, metabolism and potential importance to human health." Journal of the Science of Food and Agriculture **80**(7): 1044-1062.

Chen, H., A. Chedotal, et al. (1997). "Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III." Neuron **19**(3): 547-559.

Choi, D. Y., Y. H. Baek, et al. (2009). "Stimulatory effect of Cinnamomum cassia and cinnamic acid on angiogenesis through up-regulation of VEGF and Flk-1/KDR expression." International Immunopharmacology **9**(7-8): 959-967.

Chong, A. S. F. and C. R. Parish (1985). "Nonimmune Lymphocyte Macrophage Interaction .1. Quantification by an Automated Colorimetric Assay." Cellular Immunology **92**(2): 265-276.

Chung, A. S. and N. Ferrara (2011). "Developmental and Pathological Angiogenesis." Annual Review of Cell and Developmental Biology, Vol 27 **27**: 563-584.

Civitelli, R. (1997). "In vitro and in vivo effects of ipriflavone on bone formation and bone biomechanics." Calcified Tissue International **61**: S12-S14.

Clapp, C., S. Thebault, et al. (2009). "Peptide Hormone Regulation of Angiogenesis." Physiological Reviews **89**(4): 1177-1215.

Collinson, D. J. and R. Donnelly (2004). "Therapeutic angiogenesis in peripheral arterial disease: Can biotechnology produce an effective collateral circulation?" European Journal of Vascular and Endovascular Surgery **28**(1): 9-23.

Comalada, M., I. Ballester, et al. (2006). "Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure-activity relationship." Biochemical Pharmacology **72**(8): 1010-1021.

Conn, M. P., Ed. (2012). Methods in Cell Biology, Elsevier.

Cordell, G. A., C. W. W. Beecher, et al. (1991). "Can Ethnopharmacology Contribute to the Development of New Anticancer Drugs." Journal of Ethnopharmacology **32**(1-3): 117-133.

Cornish, S. M., P. D. Chilibeck, et al. (2009). "A randomized controlled trial of the effects of flaxseed lignan complex on metabolic syndrome composite score and bone mineral in older adults." Applied Physiology Nutrition and Metabolism-Physiologie Appliquee Nutrition Et Metabolisme **34**(2): 89-98.

Cornwell, T., W. Cohick, et al. (2004). "Dietary phytoestrogens and health." Phytochemistry **65**(8): 995-1016.

Costa, F. and R. Soares (2009). "Nicotine: A pro-angiogenic factor." Life Sciences **84**(23-24): 785-790.

Coward, L., M. Smith, et al. (1998). "Chemical modification of isoflavones in soyfoods during cooking and processing." American Journal of Clinical Nutrition **68**(6 Suppl): 1486S-1491S.

Cragg, G. M. and D. J. Newman (2001). "Natural product drug discovery in the next millennium." Pharmaceutical Biology **39**: 8-17.

Cragg, G. M. and D. J. Newman (2001). "Natural products drug discovery and development at the United States National Cancer Institute." Drug Discovery and Traditional Chinese Medicine: Science, Regulation, and Globalization: 19-32.

Cristofanilli, M., C. Charnsangavej, et al. (2002). "Angiogenesis modulation in cancer research: Novel clinical approaches." Nature Reviews Drug Discovery **1**(6): 415-426.

Cross, M. J. and L. Claesson-Welsh (2001). "FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition." Trends in Pharmacological Sciences **22**(4): 201-207.

Crozier, A., D. Del Rio, et al. (2010). "Bioavailability of dietary flavonoids and phenolic compounds." Molecular Aspects of Medicine **31**(6): 446-467.

Cullimore, J. V., R. Ranjeva, et al. (2001). "Perception of lipo-chitooligosaccharidic Nod factors in legumes." Trends in Plant Science **6**(1): 24-30.

Damiani, I., K. Morreel, et al. (2005). "Metabolite profiling reveals a role for atypical cinnamyl alcohol dehydrogenase CAD1 in the synthesis of coniferyl alcohol in tobacco xylem." Plant Molecular Biology **59**(5): 753-769.

Davis-Searles, P. R., Y. Nakanishi, et al. (2005). "Milk thistle and prostate cancer: Differential effects of pure flavonolignans from Silybum marianum on anti proliferative end points in human prostate carcinoma cells." Cancer Research **65**(10): 4448-4457.

Davis, G. D., N. J. Emenaker, et al. (2010). "Cellular Proliferation, Apoptosis and Angiogenesis: Molecular Targets for Nutritional Preemption of Cancer." Seminars in Oncology **37**(3): 243-257.

Day, A. J., F. J. Canada, et al. (2000). "Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase." FEBS Letters **468**(2-3): 166-170.

Day, A. J., M. S. DuPont, et al. (1998). "Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity." FEBS Letters **436**(1): 71-75.

Dell'Era, P., M. Mohammadi, et al. (1999). "Different tyrosine autophosphorylation requirements in fibroblast growth factor receptor-1 mediate urokinase-type

plasminogen astivator induction and mitogenesis." Molecular Biology of the Cell **10**(1): 23-33.

Dembinska-Kiec, A., A. Polus, et al. (2004). "The PPAR gamma and beta-carotene activity in the umbilical cord progenitor cell maturation during angiogenesis." European Heart Journal **25**: 471-471.

Demmig-Adams, B. and W. W. Adams (2002). "Antioxidants in photosynthesis and human nutrition." Science **298**(5601): 2149-2153.

Denarie, J., F. Debelle, et al. (1996). "Rhizobium lipo-chitooligosaccharide nodulation factors: Signalling molecules mediating recognition and morphogenesis." Annual Review of Biochemistry **65**: 503-535.

Deshane, J., S. F. Chen, et al. (2007). "Stromal cell-derived factor 1 promotes angiogenesis via a heme oxygenase 1-dependent mechanism." Journal of Experimental Medicine **204**(3): 605-618.

DeVries, J. W. (2003). "On defining dietary fibre." Proceedings of the Nutrition Society **62**(1): 37-43.

Dewhalley, C. V., S. M. Rankin, et al. (1990). "Flavonoids Inhibit the Oxidative Modification of Low-Density Lipoproteins by Macrophages." Biochemical Pharmacology **39**(11): 1743-1750.

Djordjevic, M. A., A. Bezos, et al. (2013). "Plant symbiosis signalling molecules control mammalian angiogenesis." Submitted.

Djordjevic, M. A., M. Oakes, et al. (2007). "The Glycine max xylem sap and apoplast proteome." Journal of Proteome Research **6**(9): 3771-3779.

Donovan, D., N. J. Brown, et al. (2001). "Comparison of three in vitro human 'angiogenesis' assays with capillaries formed in vivo." Angiogenesis **4**(2): 113-121.

Du Fall, L. A. (2009). Characterisation of low molecular weight bioactive plant metabolites, Australian National University, Honours Thesis.

Du, M., X. Yang, et al. (2012). "Low-dose dietary genistein negates the therapeutic effect of tamoxifen in athymic nude mice." Carcinogenesis **33**(4): 895-901.

Dugas, A. J., J. Castaneda-Acosta, et al. (2000). "Evaluation of the total peroxyl radical-scavenging capacity of flavonoids: Structure-activity relationships." Journal of Natural Products **63**(3): 327-331.

Edmunds, K. M., A. C. Holloway, et al. (2005). "The effects of dietary phytoestrogens on aromatase activity in human endometrial stromal cells." Reproduction Nutrition Development **45**(6): 709-720.

Egelhoff, T. T. and J. A. Spudich (1991). "Molecular-Genetics of Cell-Migration - Dictyostelium as a Model System." Trends in Genetics **7**(5): 161-166.

Egleton, R. D., K. C. Brown, et al. (2009). "Angiogenic activity of nicotinic acetylcholine receptors: Implications in tobacco-related vascular diseases." Pharmacology & Therapeutics **121**(2): 205-223.

Ejaz, S. and C. W. Lim (2005). "Toxicological overview of cigarette smoking on angiogenesis." Environmental Toxicology and Pharmacology **20**(2): 335-344.

Eklund, P. C., O. K. Langvik, et al. (2005). "Chemical studies on antioxidant mechanisms and free radical scavenging properties of lignans." Organic & Biomolecular Chemistry **3**(18): 3336-3347.

Elattar, T. M. A. and A. S. Virji (2000). "The inhibitory effect of curcumin, genistein, quercetin and cisplatin on the growth of oral cancer cells in vitro." Anticancer Research **20**(3A): 1733-1738.

Eliceiri, B. P. and D. A. Cheresh (2001). "Adhesion events in angiogenesis." Current Opinion in Cell Biology **13**(5): 563-568.

Erlund, I., E. Meririnne, et al. (2001). "Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice." Journal of Nutrition **131**(2): 235-241.

Eswarakumar, V. P., I. Lax, et al. (2005). "Cellular signalling by fibroblast growth factor receptors." Cytokine and Growth Factor Reviews **16**(2): 139-149.

Falkow, S. (1988). "Molecular Koch's postulates applied to microbial pathogenicity." Reviews of Infectious Diseases **10** Suppl 2: S274-276.

Faridi, A., C. Rudlowski, et al. (2002). "Long-term follow-up and prognostic significance of angiogenic basic fibroblast growth factor (bFGF) expression in patients with breast cancer." Pathology Research and Practice **198**(1): 1-5.

Feldinghabermann, B., B. M. Mueller, et al. (1992). "Involvement of Integrin Alpha-V Gene-Expression in Human-Melanoma Tumourigenicity." Journal of Clinical Investigation **89**(6): 2018-2022.

Felgines, C., O. Texier, et al. (2000). "Bioavailability of the flavanone naringenin and its glycosides in rats." American Journal of Physiology and Gastrointest Liver Physiology **279**(6): G1148-1154.

Fenton, J. I. and N. G. Hord (2004). "Flavonoids promote cell migration in nontumourigenic colon epithelial cells differing in Apc genotype: Implications of matrix metalloproteinase activity." Nutrition and Cancer-an International Journal **48**(2): 182-188.

Ferrandiz, M. L., A. G. R. Nair, et al. (1990). "Inhibition of Sheep Platelet Arachidonate Metabolism by Flavonoids from Spanish and Indian Medicinal Herbs." Pharmazie **45**(3): 206-208.

Ferrara, N. (2001). "Role of vascular endothelial growth factor in regulation of physiological angiogenesis." American Journal of Physiology-Cell Physiology **280**(6): C1358-C1366.

Ferrara, N. (2002). "VEGF and the quest for tumour angiogenesis factors." Nature Reviews Cancer **2**(10): 795-803.

Ferrara, N. (2004). "Vascular endothelial growth factor: Basic science and clinical progress." Endocrine Reviews **25**(4): 581-611.

Ferrara, N. (2009). "VEGF-A: a critical regulator of blood vessel growth." European Cytokine Network **20**(4): 158-163.

Ferrara, N., H. Chen, et al. (1998). "Vascular endothelial growth factor is essential for corpus luteum angiogenesis." Nature Medicine **4**(3): 336-340.

Ferrara, N., H. P. Gerber, et al. (2003). "The biology of VEGF and its receptors." Nature Medicine **9**(6): 669-676.

Filardo, E. J., P. C. Brooks, et al. (1995). "Requirement of the Npxy Motif in the Integrin Beta-3 Subunit Cytoplasmic Tail for Melanoma Cell-Migration in-Vitro and in-Vivo." Journal of Cell Biology **130**(2): 441-450.

Folkman, J. (1993). "Clinical-Applications of Angiogenesis Research." Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology **6**(6): 686-686.

Folkman, J. (2000). "Lessons from antiangiogenic therapy of cancer patients." Clinical Cancer Research **6**: 4483s-4483s.

Folkman, J. (2007). "Opinion - Angiogenesis: an organizing principle for drug discovery?" Nature Reviews Drug Discovery **6**(4): 273-286.

Folkman, J., M. Bach, et al. (1971). "Tumour Angiogenesis - Therapeutic Implications." New England Journal of Medicine **285**(21): 1182-1186.

Font, M. A., A. Arboix, et al. (2010). "Angiogenesis, neurogenesis and neuroplasticity in ischemic stroke." Current Cardiology Reviews **6**(3): 238-244.

Formica, J. V. and W. Regelson (1995). "Review of the biology of quercetin and related bioflavonoids." Food and Chemical Toxicology **33**(12): 1061-1080.

Fotsis, T., M. Pepper, et al. (1993). "Genistein, a Dietary-Derived Inhibitor of Invitro Angiogenesis." Proceedings of the National Academy of Sciences of the United States of America **90**(7): 2690-2694.

Fotsis, T., M. Pepper, et al. (1995). "Genistein, a Dietary Ingested Isoflavonoid, Inhibits Cell-Proliferation and in-Vitro Angiogenesis." Journal of Nutrition **125**(3): S790-S797.

Fotsis, T., M. S. Pepper, et al. (1997). "Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis." Cancer Research **57**(14): 2916-2921.

Friedrichs, W., S. B. Ruparel, et al. (2011). "Omega-3 Fatty Acid Inhibition of Prostate Cancer Progression to Hormone Independence Is Associated With Suppression

of mTOR Signalling and Androgen Receptor Expression." Nutrition and Cancer-an International Journal **63**(5): 771-777.

Fukuda, S., S. Kaga, et al. (2006). "Resveratrol ameliorates myocardial damage by inducing vascular endothelial growth factor-angiogenesis and tyrosine kinase receptor Flk-1." Cell Biochemistry and Biophysics **44**(1): 43-49.

Fukumitsu, S., K. Aida, et al. (2010). "Flaxseed lignan lowers blood cholesterol and decreases liver disease risk factors in moderately hypercholesterolemic men." Nutrition Research **30**(7): 441-446.

Galbraith, S. M., D. J. Chaplin, et al. (2001). "Effects of combretastatin A4 phosphate on endothelial cell morphology in vitro and relationship to tumour vascular targeting activity in vivo." Anticancer Research **21**(1A): 93-102.

Galluzzo, P., P. Ascenzi, et al. (2008). "The nutritional flavanone naringenin triggers antiestrogenic effects by regulating estrogen receptor alpha-palmitoylation." Endocrinology **149**(5): 2567-2575.

Garbisa, S., S. Biggin, et al. (1999). "Tumour invasion: molecular shears blunted by green tea." Nature Medicine **5**(11): 1216-1216.

Garbisa, S., L. Sartor, et al. (2001). "Tumour gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate." Cancer **91**(4): 822-832.

Gardana, C., S. Guarnieri, et al. (2007). "Flavanone plasma pharmacokinetics from blood orange juice in human subjects." British Journal of Nutrition **98**(1): 165-172.

Gee, J. M., M. S. DuPont, et al. (2000). "Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway." Journal of Nutrition **130**(11): 2765-2771.

Gennari, C., D. Agnusdei, et al. (1998). "Effect of ipriflavone - a synthetic derivative of natural isoflavones - On bone mass loss in the early years after menopause." Menopause-the Journal of the North American Menopause Society **5**(1): 9-15.

Goodwin, A. M. (2007). "In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents." Microvascular Research **74**(2-3): 172-183.

Gordaliza, M. (2007). "Natural products as leads to anticancer drugs." Clinical & Translational Oncology **9**(12): 767-776.

Grant, D. S., P. L. Lelkes, et al. (1991). "Intracellular Mechanisms Involved in Basement-Membrane Induced Blood-Vessel Differentiation Invitro." In Vitro Cellular and Developmental Biology **27**(4): 327-336.

Griffioen, A. W. and G. Molema (2000). "Angiogenesis: Potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation." Pharmacological Reviews **52**(2): 237-268.

Gulcin, I., R. Elias, et al. (2006). "Antioxidant activity of lignans from fringe tree (Chionanthus virginicus L.)." European Food Research and Technology **223**(6): 759-767.

Gururaj, A. E., M. Belakavadi, et al. (2002). "Molecular mechanisms of anti-angiogenic effect of curcumin." Biochemical and Biophysical Research Communications **297**(4): 934-942.

Hallund, J., I. Tetens, et al. (2008). "The effect of a lignan complex isolated from flaxseed on inflammation markers in healthy postmenopausal women." Nutrition Metabolism and Cardiovascular Diseases **18**(7): 497-502.

Hamilton, G. R. and T. F. Baskett (2000). "In the arms of Morpheus: the development of morphine for postoperative pain relief." Canadian Journal of Anaesthesia-Journal Canadien de Anesthesie **47**(4): 367-374.

Han, H. Y., X. H. Wang, et al. (2008). "Lignans isolated from Campylotropis hirtella (Franch.) Schindl. decreased prostate specific antigen and androgen receptor expression in LNCaP cells." Journal of Agricultural and Food Chemistry **56**(16): 6928-6935.

Harborne, J. B. and C. A. Williams (2000). "Advances in flavonoid research since 1992." Phytochemistry **55**(6): 481-504.

Harris, D. M., E. Besselink, et al. (2005). "Phytoestrogens induce differential estrogen receptor alpha- or beta-mediated responses in transfected breast cancer cells." Experimental Biology and Medicine **230**(8): 558-568.

Harvey, A. L. (2008). "Natural products in drug discovery." Drug Discovery Today **13**(19-20): 894-901.

Haskard, D. O., S. Strobel, et al. (1989). "Mechanisms of Lymphocyte Adhesion to Endothelial-Cells - Studies Using a Lfa-1-Deficient Cell-Line." Immunology **66**(1): 111-116.

Hausott, B., H. Greger, et al. (2003). "Naturally occurring lignans efficiently induce apoptosis in colorectal tumour cells." Journal of Cancer Research and Clinical Oncology **129**(10): 569-576.

Havsteen, B. H. (2002). "The biochemistry and medical significance of the flavonoids." Pharmacology and Therapeutics **96**(2-3): 67-202.

Hegarty, V. M., H. M. May, et al. (2000). "Tea drinking and bone mineral density in older women." American Journal of Clinical Nutrition **71**(4): 1003-1007.

Heinonen, S., T. Nurmi, et al. (2001). "In vitro metabolism of plant lignans: New precursors of mammalian lignans enterolactone and enterodiol." Journal of Agricultural and Food Chemistry **49**(7): 3178-3186.

Heo, H. J., D. O. Kim, et al. (2004). "Effect of antioxidant flavanone, naringenin, from Citrus junos on neuroprotection." Journal of Agricultural and Food Chemistry **52**(6): 1520-1525.

Herridge, D. F. (1997). Carbon and nitrogen nutrition of two annual legumes, University of Western Australia. **PhD**.

Hertog, M. G. L., E. J. M. Feskens, et al. (1993). "Dietary Antioxidant Flavonoids and Risk of Coronary Heart-Disease - the Zutphen Elderly Study." Lancet **342**(8878): 1007-1011.

Hirano, T., K. Abe, et al. (1995). "Citrus Flavone Tangeretin Inhibits Leukemic Hl-60 Cell-Growth Partially through Induction of Apoptosis with Less Cytotoxicity on Normal Lymphocytes." British Journal of Cancer **72**(6): 1380-1388.

Hoeben, A., B. Landuyt, et al. (2004). "Vascular endothelial growth factor and angiogenesis." Pharmacological Reviews **56**(4): 549-580.

Hollman, P. C. H. (2004). "Absorption, bioavailability, and metabolism of flavonoids." Pharmaceutical Biology **42**: 74-83.

Hollman, P. C. H. and M. B. Katan (1999). "Health effects and bioavailability of dietary flavonols." Free Radical Research **31**: S75-S80.

Holt, G. A. and A. Chandra (2002). "Herbs in the modern healthcare environment - An overview of uses, legalities, and the role of the healthcare professional." Clinical Research and Regulatory Affairs **19**(1): 83-107.

Hood, J. D. and D. A. Cheresh (2002). "Role of integrins in cell invasion and migration." Nature Reviews Cancer **2**(2): 91-100.

Hooper, L., P. A. Kroon, et al. (2008). "Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials." American Journal of Clinical Nutrition **88**(1): 38-50.

Hoult, J. R., M. A. Moroney, et al. (1994). "Actions of flavonoids and coumarins on lipoxygenase and cyclooxygenase." Methods Enzymology **234**: 443-454.

Howitz, K. T. and D. A. Sinclair (2008). "Xenohormesis: sensing the chemical cues of other species." Cell **133**(3): 387-391.

Itokawa, H., S. L. Morris-Natschke, et al. (2008). "Plant-derived natural product research aimed at new drug discovery." Journal of Natural Medicines **62**(3): 263-280.

Jagetia, A., G. C. Jagetia, et al. (2007). "Naringin, a grapefruit flavanone, protects V79 cells against the bleomycin-induced genotoxicity and decline in survival." Journal of Applied Toxicology **27**(2): 122-132.

Jain, R. K., D. G. Duda, et al. (2006). "Lessons from phase III clinical trials on anti-VEGF therapy for cancer." Nature Clinical Practice Oncology **3**(1): 24-40.

Jain, R. K., K. Schlenger, et al. (1997). "Quantitative angiogenesis assays: Progress and problems." Nature Medicine **3**(11): 1203-1208.

Javerzat, S., P. Auguste, et al. (2002). "The role of fibroblast growth factors in vascular development." Trends in Molecular Medicine **8**(10): 483-489.

Jenab, M. and L. U. Thompson (1996). "The influence of flaxseed and lignans on colon carcinogenesis and beta-glucuronidase activity." Carcinogenesis **17**(6): 1343-1348.

Jiang, C., R. Agarwal, et al. (2000). "Anti-angiogenic potential of a cancer chemopreventive flavonoid antioxidant, silymarin: inhibition of key attributes of vascular endothelial cells and angiogenic cytokine secretion by cancer epithelial cells." Biochemical and Biophysical Research Communications **276**(1): 371-378.

Ju, Y. H., D. R. Doerge, et al. (2002). "Dietary genistein negates the inhibitory effect of tamoxifen on growth of estrogen-dependent human breast cancer (MCF-7) cells implanted in athymic mice." Cancer Research **62**(9): 2474-2477.

Jung, J. W., I. H. Park, et al. (2012). "Naringenin Inhibits Extracellular Matrix Production via Extracellular Signal-Regulated Kinase Pathways in Nasal Polyp-Derived Fibroblasts." Phytotherapy Research.

Kaga, S., L. J. Zhan, et al. (2005). "Resveratrol enhances neovascularization in the infarcted rat myocardium through the induction of thioredoxin-1, heme oxygenase-1 and vascular endothelial growth factor." Journal of Molecular and Cellular Cardiology **39**(5): 813-822.

Kale, A., S. Gawande, et al. (2008). "Cancer phytotherapeutics: Role for flavonoids at the cellular level." Phytotherapy Research **22**(5): 567-577.

Kanadaswami, C., L. T. Lee, et al. (2005). "The antitumour activities of flavonoids." In Vivo **19**(5): 895-909.

Kancheva, V. D., L. Saso, et al. (2012). "Antiradical and antioxidant activities of new bio-antioxidants." Biochimie **94**(2): 403-415.

Katiyar, C., A. Gupta, et al. (2012). "Drug discovery from plant sources: An integrated approach." Ayu **33**(1): 10-19.

Kaufman, P. B., J. A. Duke, et al. (1997). "A comparative survey of leguminous plants as sources of the isoflavones, genistein and daidzein: implications for human nutrition and health." Journal of Alternative and Complementary Medicine **3**(1): 7-12.

Kawaii, S., Y. Tomono, et al. (1999). "Antiproliferative activity of flavonoids on several cancer cell lines." Bioscience Biotechnology and Biochemistry **63**(5): 896-899.

Kerbel, R. S. (1997). "A cancer therapy resistant to resistance." Nature **390**(6658): 335-336.

Khurana, R., M. Simons, et al. (2005). "Role of angiogenesis in cardiovascular disease - A critical appraisal." Circulation **112**(12): 1813-1824.

King, R. A. and D. B. Bursill (1998). "Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans." American Journal of Clinical Nutrition **67**(5): 867-872.

Kingston, D. G. (2011). "Modern natural products drug discovery and its relevance to biodiversity conservation." Journal of Natural Products **74**(3): 496-511.

Kirk, E. A., P. Sutherland, et al. (1998). "Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice." Journal of Nutrition **128**(6): 954-959.

Klein, S., A. Bikfalvi, et al. (1996). "Integrin regulation by endogenous expression of 18-kDa fibroblast growth factor-2." Journal of Biological Chemistry **271**(37): 22583-22590.

Koehn, F. E. and G. T. Carter (2005). "The evolving role of natural products in drug discovery." Nature Reviews Drug Discovery **4**(3): 206-220.

Krause, M. and R. Galensa (1992). "Determination of Naringenin and Naringenin-Chalcone in Tomato Skins by Reversed Phase Hplc after Solid-Phase Extraction." Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung **194**(1): 29-32.

Kris-Etherton, P. M., M. Lefevre, et al. (2004). "Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis." Annual Review of Nutrition **24**: 511-538.

Krishnan, H. B., S. S. Natarajan, et al. (2011). "Protein and metabolite composition of xylem sap from field-grown soybeans (Glycine max)." Planta **233**(5): 921-931.

Kuiper, G. G. J. M., J. G. Lemmen, et al. (1998). "Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta." Endocrinology **139**(10): 4252-4263.

Kushiro, T., E. Nambara, et al. (2003). "The key to signalling." Nature **422**(6928): 122-122.

Lackie, J. M. and D. D. Bono (1977). "Interactions of Neutrophil Granulocytes (Pmns) and Endothelium Invitro." Microvascular Research **13**(1): 107-112.

Lah, J. J., W. Cui, et al. (2007). "Effects and mechanisms of silibinin on human hepatoma cell lines." World Journal of Gastroenterology **13**(40): 5299-5305.

Lakshman, M., L. Xu, et al. (2008). "Dietary genistein inhibits metastasis of human prostate cancer in mice." Cancer Research **68**(6): 2024-2032.

Lam, I. K., D. Alex, et al. (2012). "In vitro and in vivo structure and activity relationship analysis of polymethoxylated flavonoids: identifying sinensetin as a novel antiangiogenesis agent." Molecular Nutrition and Food Research **56**(6): 945-956.

Lamalice, L., F. Le Boeuf, et al. (2007). "Endothelial cell migration during angiogenesis." Circulation Research **100**(6): 782-794.

Landberg, R., S. O. Andersson, et al. (2010). "Rye Whole Grain and Bran Intake Compared with Refined Wheat Decreases Urinary C-Peptide, Plasma Insulin, and Prostate Specific Antigen in Men with Prostate Cancer." Journal of Nutrition **140**(12): 2180-2186.

Laughton, M. J., P. J. Evans, et al. (1991). "Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability." Biochemical Pharmacology **42**(9): 1673-1681.

Lee, C. H., T. S. Jeong, et al. (2001). "Anti-atherogenic effect of citrus flavonoids, naringin and naringenin, associated with hepatic ACAT and aortic VCAM-1 and MCP-1 in high cholesterol-fed rabbits." Biochemical and Biophysical Research Communications **284**(3): 681-688.

Lee, D. Y., M. C. Song, et al. (2007). "Lignans from the fruits of Cornus kousa Burg. and their cytotoxic effects on human cancer cell lines." Archives of Pharmacal Research **30**(4): 402-407.

Lee, K. H. and E. M. Choi (2006). "Stimulatory effects of extract prepared from the bark of Cinnamomum cassia blume on the function of osteoblastic MC3T3-E1 cells." Phytotherapy Research **20**(11): 952-960.

Lee, M. J., J. D. Lambert, et al. (2004). "Delivery of tea polyphenols to the oral cavity by green tea leaves and black tea extract." Cancer Epidemiol Biomarkers Prev **13**(1): 132-137.

Leeson, P. D. and B. Springthorpe (2007). "The influence of drug-like concepts on decision-making in medicinal chemistry." Nature Reviews Drug Discovery **6**(11): 881-890.

Lentini, A., C. Forni, et al. (2007). "Enhancement of transglutaminase activity and polyamine depletion in B16-F10 melanoma cells by flavonoids naringenin and hesperitin correlate to reduction of the in vivo metastatic potential." Amino Acids **32**(1): 95-100.

Lerouge, P., P. Roche, et al. (1990).."Symbiotic Host-Specificity of Rhizobium-Meliloti Is Determined by a Sulfated and Acylated Glucosamine Oligosaccharide Signal." Nature **344**(6268): 781-784.

Li, J. W. H. and J. C. Vederas (2009). "Drug Discovery and Natural Products: End of an Era or an Endless Frontier?" Science **325**(5937): 161-165.

Li, W. W., V. W. Li, et al. (2012). "Tumour Angiogenesis as a Target for Dietary Cancer Prevention." Journal of Oncology **2012**.

Liao, J., G. Y. Yang, et al. (2004). "Inhibition of lung carcinogenesis and effects on angiogenesis and apoptosis in A/J mice by oral administration of green tea." Nutrition and Cancer-an International Journal **48**(1): 44-53.

Liekens, S., E. De Clercq, et al. (2001). "Angiogenesis: regulators and clinical applications." Biochemical Pharmacology **61**(3): 253-270.

Lin, C. M., J. H. Chiu, et al. (2010). "Ferulic acid augments angiogenesis via VEGF, PDGF and HIF-1 alpha." Journal of Nutritional Biochemistry **21**(7): 627-633.

Lin, P. Y. and H. M. Lai (2006). "Bioactive compounds in legumes and their germinated products." Journal of Agricultural and Food Chemistry **54**(11): 3807-3814.

Lipinski, C. A. (2004). "Lead- and drug-like compounds: the rule-of-five revolution." Drug Discovery Today: Technologies **1**(4): 337-341.

Lipinski, C. A., F. Lombardo, et al. (2001). "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings." Advanced Drug Delivery Reviews **46**(1-3): 3-26.

Littleton, J., T. Rogers, et al. (2005). "Novel approaches to plant drug discovery based on high throughput pharmacological screening and genetic manipulation." Life Sciences **78**(5): 467-475.

Liu, C. J., P. H. Lee, et al. (2009). "Heparanase inhibitor PI-88 as adjuvant therapy for hepatocellular carcinoma after curative resection: A randomized phase II trial for safety and optimal dosage." Journal of Hepatology **50**(5): 958-968.

Liu, X. J., W. Wang, et al. (2006). "Smad3 specific inhibitor, naringenin, decreases the expression of extracellular matrix induced by TGF-beta 1 in cultured rat hepatic stellate cells." Pharmaceutical Research **23**(1): 82-89.

Lourith, N., T. Katayama, et al. (2005). "Stereochemistry and biosynthesis of 8-0-4 ' neolignans in Eucommia ulmoides: diastereoselective formation of guaiacylglycerol-8-0-4 '-(sinapyl alcohol) ether." Journal of Wood Science **51**(4): 370-378.

Lu, H. T., J. X. Li, et al. (2006). "Molecular mechanisms involved in chemoprevention of black raspberry extracts: From transcription factors to their target genes." Nutrition and Cancer-an International Journal **54**(1): 69-78.

Ma, C. Y., H. J. Zhang, et al. (2006). "Antimalarial compounds from Grewia bilamellata." Journal of Natural Products **69**(3): 346-350.

Mabjeesh, N. J., M. T. Willard, et al. (2003). "Dibenzoylmethane, a natural dietary compound, induces HIF-1 alpha and increases expression of VEGF." Biochemical and Biophysical Research Communications **303**(1): 279-286.

Macrae, W. D. and G. H. N. Towers (1984). "Biological-Activities of Lignans." Phytochemistry **23**(6): 1207-1220.

Mahdi, J. G., A. J. Mahdi, et al. (2006). "The historical analysis of aspirin discovery, its relation to the willow tree and antiproliferative and anticancer potential." Cell Proliferation **39**(2): 147-155.

Maher, C. A., C. Kumar-Sinha, et al. (2009). "Transcriptome sequencing to detect gene fusions in cancer." Nature **458**(7234): 97-101.

Manach, C., C. Morand, et al. (2003). "Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice." European Journal of Clinical Nutrition **57**(2): 235-242.

Manach, C., A. Scalbert, et al. (2004). "Polyphenols: food sources and bioavailability." American Journal of Clinical Nutrition **79**(5): 727-747.

Manach, C., G. Williamson, et al. (2005). "Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies." The American Journal of Clinical Nutrition **81**(1 Suppl): 230S-242S.

Mander, L. and H.-W. B. Liu (2010). Comprehensive Natural Product II Chemistry and Biology. Italy, Elsevier.

Manthey, J. A. and N. Guthrie (2002). "Antiproliferative activities of citrus flavonoids against six human cancer cell lines." Journal of Agricultural and Food Chemistry **50**(21): 5837-5843.

Martinez, C., J. Yanez, et al. (2003). "Effects of several polyhydroxylated flavonoids on the growth of B16F10 melanoma and Melan-a melanocyte cell lines: influence of the sequential oxidation state of the flavonoid skeleton." Melanoma Research **13**(1): 3-9.

Mathiesen, L., K. E. Malterud, et al. (1997). "Hydrogen bond-formation as basis for radical scavenging activity: A structure-activity study of C-methylated dihydrochalcones from Myrica gale and structurally related acetophenones." Free Radical Biology and Medicine **22**(1-2): 307-311.

Matou, S., D. Helley, et al. (2002). "Effect of fucoidan on fibroblast growth factor-2-induced angiogenesis in vitro." Thrombosis Research **106**(4-5): 213-221.

Matsumoto, H., Y. Ikoma, et al. (2004). "Identification and quantification of the conjugated metabolites derived from orally administered hesperidin in rat plasma." Journal of Agricultural and Food Chemistry **52**(21): 6653-6659.

Mayr, G. W., S. Windhorst, et al. (2005). "Antiproliferative plant and synthetic polyphenolics are specific inhibitors of vertebrate inositol-1,4,5-trisphosphate 3-kinases and inositol polyphosphate multikinase." Journal of Biological Chemistry **280**(14): 13229-13240.

Mazur, W. and H. Adlercreutz (2000). "Overview of naturally occurring endocrine-active substances in the human diet in relation to human health." Nutrition **16**(7-8): 654-658.

Mazur, W. M., J. A. Duke, et al. (1998). "Isoflavonoids and lignans in legumes: Nutritional and health aspects in humans." Journal of Nutritional Biochemistry **9**(4): 193-200.

McCann, M. J., C. I. R. Gill, et al. (2005). "Role of mammalian Lignans in the prevention and treatment of prostate cancer." Nutrition and Cancer International Journal **52**(1): 1-14.

McCann, S. E., J. L. Freudenheim, et al. (2003). "Risk of human ovarian cancer is related to dietary intake of selected nutrients, phytochemicals and food groups." Journal of Nutrition **133**(6): 1937-1942.

McCann, S. E., K. C. Hootman, et al. (2012). "Dietary Intakes of Total and Specific Lignans Are Associated with Clinical Breast Tumour Characteristics." Journal of Nutrition **142**(1): 91-98.

McChesney, J. D., S. K. Venkataraman, et al. (2007). "Plant natural products: Back to the future or into extinction?" Phytochemistry **68**(14): 2015-2022.

Mcfaul, S. J. and P. D. Bowman (1990). "Quantitation of Polymorphonuclear Leukocyte Adherence to Endothelial-Cells by Electronic Particle-Size Discrimination." Journal of Immunological Methods **130**(2): 171-176.

Mennen, L. I., D. Sapinho, et al. (2008). "Urinary excretion of 13 dietary flavonoids and phenolic acids in free-living healthy subjects - variability and possible use as biomarkers of polyphenol intake." European Journal of Clinical Nutrition **62**(4): 519-525.

Merz-Demlow, B. E., A. M. Duncan, et al. (2000). "Soy isoflavones improve plasma lipids in normocholesterolemic, premenopausal women." American Journal of Clinical Nutrition **71**(6): 1462-1469.

Middleton, E., C. Kandaswami, et al. (2000). "The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer." Pharmacological Reviews **52**(4): 673-751.

Milder, I. E. J., E. J. M. Feskens, et al. (2005). "Intake of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in Dutch men and women." Journal of Nutrition **135**(5): 1202-1207.

Mohan, R., J. Sivak, et al. (2000). "Curcuminoids inhibit the angiogenic response stimulated by fibroblast growth factor-2, including expression of matrix metalloproteinase gelatinase B." Journal of Biological Chemistry **275**(14): 10405-10412.

Monasterio, A., M. C. Urdaci, et al. (2004). "Flavonoids induce apoptosis in human leukemia U937 cells through caspase- and caspase-calpain-dependent pathways." Nutrition and Cancer International Journal **50**(1): 90-100.

Montanari, A., J. Chen, et al. (1998). "Citrus flavonoids: A review of past biological activity against disease - Discovery of new flavonoids from dancy tangerine cold pressed peel oil solids and leaves." Flavonoids in the Living System **439**: 103-116.

Morimoto, N., S. Takemoto, et al. (2008). "Nicotine at a low concentration promotes wound healing." Journal of Surgical Research **145**(2): 199-204.

Moroney, M. A., M. J. Alcaraz, et al. (1988). "Selectivity of Neutrophil 5-Lipoxygenase and Cyclo-Oxygenase Inhibition by an Anti-Inflammatory Flavonoid Glycoside and Related Aglycone Flavonoids." Journal of Pharmacy and Pharmacology **40**(11): 787-792.

Morreel, K., O. Dima, et al. (2010). "Mass Spectrometry-Based Sequencing of Lignin Oligomers." Plant Physiology **153**(4): 1464-1478.

Morreel, K., H. Kim, et al. (2010). "Mass Spectrometry-Based Fragmentation as an Identification Tool in Lignomics." Analytical Chemistry **82**(19): 8095-8105.

Morreel, K., J. Ralph, et al. (2004). "Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem." Plant Physiology **136**(3): 3537-3549.

Morton, M. S., O. Arisaka, et al. (2002). "Phytoestrogen concentrations in serum from Japanese men and women over forty years of age." Journal of Nutrition **132**(10): 3168-3171.

Moscatelli, D., M. Presta, et al. (1986). "Purification of a Factor from Human-Placenta That Stimulates Capillary Endothelial-Cell Protease Production, DNA-Synthesis, and Migration." Proceedings of the National Academy of Sciences of the United States of America **83**(7): 2091-2095.

Mourao, L. R. M. B., R. S. S. Santana, et al. (2009). "Protective action of indole-3-acetic acid on induced hepatocarcinoma in mice." Cell Biochemistry and Function **27**(1): 16-22.

Mousa, S. and S. A. Mousa (2006). "Cellular and molecular mechanisms of nicotine's pro-angiogenesis activity and its potential impact on cancer." Journal of Cellular Biochemistry **97**(6): 1370-1378.

Mulvihill, E. E. and M. W. Huff (2010). "Antiatherogenic properties of flavonoids: Implications for cardiovascular health." Canadian Journal of Cardiology **26**: 17a-21a.

Murkies, A. L., C. Lombard, et al. (2008). "Dietary flour supplementation decreases post-menopausal hot flushes: Effect of soy and wheat (Reprinted from Maturitas, vol 21, pg 189-195, 1995)." Maturitas **61**(1-2): 27-33.

Murphy, S., B. Larrivee, et al. (2006). "Identification of sokotrasterol sulfate as a novel proangiogenic steroid." Circulation Research **99**(3): 257-265.

Nestel, P. J., S. Pomeroy, et al. (1999). "Isoflavones from red clover improve systemic arterial compliance but not plasma lipids in menopausal women." Journal of Clinical Endocrinology and Metabolism **84**(3): 895-898.

Neuhouser, M. L. (2004). "Dietary flavonoids and cancer risk: Evidence from human population studies." Nutrition and Cancer International Journal **50**(1): 1-7.

New, D. C., D. M. Miller-Martini, et al. (2003). "Reporter gene assays and their applications to bioassays of natural products." Phytotherapy Research **17**(5): 439-448.

Newman, D. J. (2008). "Natural products as leads to potential drugs: An old process or the new hope for drug discovery?" Journal of Medicinal Chemistry **51**(9): 2589-2599.

Newman, D. J. (2011). "Natural products as sources of and leads to drugs." Abstracts of Papers of the American Chemical Society **241**.

Newman, D. J. and G. M. Cragg (2007). "Natural products as sources of new drugs over the last 25 years." Journal of Natural Products **70**(3): 461-477.

Newman, D. J. and G. M. Cragg (2012). "Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010." Journal of Natural Products **75**(3): 311-335.

Nicosia, R. F. and A. Ottinetti (1990). "Growth of Microvessels in Serum-Free Matrix Culture of Rat Aorta - a Quantitative Assay of Angiogenesis Invitro." Laboratory Investigation **63**(1): 115-122.

Nijveldt, R. J., E. van Nood, et al. (2001). "Flavonoids: a review of probable mechanisms of action and potential applications." American Journal of Clinical Nutrition **74**(4): 418-425.

Nijveldt, R. J., E. van Nood, et al. (2001). "Flavonoids: a review of probable mechanisms of action and potential applications." American Journal of Clinical Nutrition **74**(4): 418-425.

Njuguna, N. M., C. Masimirembwa, et al. (2012). "Identification and Characterization of Reactive Metabolites in Natural Products-Driven Drug Discovery." Journal of Natural Products **75**(3): 507-513.

Novaes, E., M. Kirst, et al. (2010). "Lignin and Biomass: A Negative Correlation for Wood Formation and Lignin Content in Trees." Plant Physiology **154**(2): 555-561.

Nowak, D. G., J. Woolard, et al. (2008). "Expression of pro- and anti-angiogenic isoforms of VEGF is differentially regulated by splicing and growth factors." Journal of Cell Science **121**(20): 3487-3495.

Nugent, M. A. and R. V. Iozzo (2000). "Fibroblast growth factor-2." International Journal of Biochemistry and Cell Biology **32**(2): 115-120.

O' Neill, H. C. and C. R. Parish (1983). "A Rapid, Automated Colorimetric Assay for Measuring Antibody-Binding to Cell-Surface Antigens." Journal of Immunological Methods **64**(3): 257-268.

Oikawa, T., M. Shimamura, et al. (1992). "Inhibition of angiogenesis by staurosporine, a potent protein kinase inhibitor." J Antibiot (Tokyo) **45**(7): 1155-1160.

Ornitz, D. M. and N. Itoh (2001). "Fibroblast growth factors." Genome Biology **2**(3).

Ortuno, A., D. Garciapuig, et al. (1995). "Flavanone and Nootkatone Levels in Different Varieties of Grapefruit and Pomelo." Journal of Agricultural and Food Chemistry **43**(1): 1-5.

Osman, H. E., N. Maalej, et al. (1998). "Grape juice but not orange or grapefruit juice inhibits platelet activity in dogs and monkeys (Macaca fasciularis)." Journal of Nutrition **128**(12): 2307-2312.

Otrock, Z. K., R. A. R. Mahfouz, et al. (2007). "Understanding the biology of angiogenesis: Review of the most important molecular mechanisms." Blood Cells Molecules and Diseases **39**(2): 212-220.

Pan, B., H. Ren, et al. (2012). "Hypochlorite-induced oxidative stress elevates the capability of HDL in promoting breast cancer metastasis." Journal of Translational Medicine **10**(65).

Pandi-Perumal, S. R., V. Srinivasan, et al. (2006). "Melatonin - Nature's most versatile biological signal?" FEBS Journal **273**(13): 2813-2838.

Pandya, N. M., N. S. Dhalla, et al. (2006). "Angiogenesis - a new target for future therapy." Vascular Pharmacology **44**(5): 265-274.

Paper, D. H. (1998). "Natural products as angiogenesis inhibitors." Planta Medica **64**(8): 686-695.

Papetti, M. and I. M. Herman (2002). "Mechanisms of normal and tumour-derived angiogenesis." American Journal of Physiology-Cell Physiology **282**(5): C947-C970.

Parish, C. R., C. Freeman, et al. (1999). "Identification of sulfated oligosaccharide-based inhibitors of tumour growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity." Cancer Research **59**(14): 3433-3441.

Passamonti, S., M. Terdoslavich, et al. (2009). "Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organisms." Current Drug Metabolism **10**(4): 369-394.

Pavese, J. M., R. L. Farmer, et al. (2010). "Inhibition of cancer cell invasion and metastasis by genistein." Cancer and Metastasis Reviews **29**(3): 465-482.

Penalvo, J. L., S. M. Heinonen, et al. (2004). "Plant lignans in soy-based health supplements." Journal of Agricultural and Food Chemistry **52**(13): 4133-4138.

Pepper, M. S. (1997). "Manipulating angiogenesis - From basic science to the bedside." Arteriosclerosis Thrombosis and Vascular Biology **17**(4): 605-619.

Perez-Vizcaino, F., J. Duarte, et al. (2012). "The flavonoid paradox: conjugation and deconjugation as key steps for the biological activity of flavonoids." Journal of the Science of Food and Agriculture **92**(9): 1822-1825.

Peterson, G. and S. Barnes (1991). "Genistein Inhibition of the Growth of Human Breast-Cancer Cells - Independence from Estrogen-Receptors and the Multidrug Resistance Gene." Biochemical and Biophysical Research Communications **179**(1): 661-667.

Peterson, J., J. Dwyer, et al. (2010). "Dietary lignans: physiology and potential for cardiovascular disease risk reduction." Nutrition Reviews **68**(10): 571-603.

Pezzuto, J. M. (1997). "Plant-derived anticancer agents." Biochemical Pharmacology **53**(2): 121-133.

Piggott, A. M. and P. Karuso (2004). "Quality, not quantity: the role of natural products and chemical proteomics in modern drug discovery." Combinatorial Chemistry and High Throughput Screening **7**(7): 607-630.

Polnaszek, N., B. Kwabi-Addo, et al. (2003). "Fibroblast growth factor 2 promotes tumour progression in an autochthonous mouse model of prostate cancer." Cancer Research **63**(18): 5754-5760.

Potterat, O. and M. Hamburger (2006). "Natural products in drug discovery - Concepts and approaches for tracking bioactivity." Current Organic Chemistry **10**(8): 899-920.

Powers, C. J., S. W. McLeskey, et al. (2000). "Fibroblast growth factors, their receptors and signalling." Endocrine-Related Cancer **7**(3): 165-197.

Prasad, K. (2009). "Flaxseed and Cardiovascular Health." Journal of Cardiovascular Pharmacology **54**(5): 369-377.

Presta, M., P. Dell'Era, et al. (2005). "Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis." Cytokine and Growth Factor Reviews **16**(2): 159-178.

Presta, M., L. Tiberio, et al. (1991). "Basic Fibroblast Growth-Factor Requires a Long-Lasting Activation of Protein-Kinase-C to Induce Cell-Proliferation in Transformed Fetal Bovine Aortic Endothelial-Cells." Cell Regulation **2**(9): 719-726.

Prosky, L. (2000). "When is dietary fiber considered a functional food?" Biofactors **12**(1-4): 289-297.

Qi, W. T., C. R. Weber, et al. (2011). "Genistein inhibits proliferation of colon cancer cells by attenuating a negative effect of epidermal growth factor on tumour suppressor FOXO3 activity." BMC Cancer **11**:219.

Qin, L., L. T. Jin, et al. (2011). "Naringenin reduces lung metastasis in a breast cancer resection model." Protein and Cell **2**(6): 507-516.

Qu, H. Y., R. L. Madl, et al. (2005). "Lignans are involved in the antitumour activity of wheat bran in colon cancer SW480 cells." Journal of Nutrition **135**(3): 598-602.

Ramasamy, K. and R. Agarwal (2008). "Multitargeted therapy of cancer by silymarin." Cancer Letters **269**(2): 352-362.

Raskin, I., D. M. Ribnicky, et al. (2002). "Plants and human health in the twenty-first century." Trends in Biotechnology **20**(12): 522-531.

Ratty, A. K. and N. P. Das (1988). "Effects of Flavonoids on Nonenzymatic Lipid-Peroxidation - Structure-Activity Relationship." Biochemical Medicine and Metabolic Biology **39**(1): 69-79.

Ren, W. Y., Z. H. Qiao, et al. (2003). "Flavonoids: Promising anticancer agents." Medicinal Research Reviews **23**(4): 519-534.

Ribatti, D., M. Presta, et al. (1999). "Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo." Blood **93**(8): 2627-2636.

Rice-Evans, C. (2004). "Flavonoids and isoflavones: absorption, metabolism, and bioactivity." Free Radical Biology in Medicine **36**(7): 827-828.

Rishton, G. M. (2008). "Natural products as a robust source of new drugs and drug leads: Past successes and present day issues." American Journal of Cardiology **101**(10A): 43d-49d.

Rodriguez, J., J. Yanez, et al. (2002). "Effects of several flavonoids on the growth of B16F10 and SK-MEL-1 melanoma cell lines: relationship between structure and activity." Melanoma Research **12**(2): 99-107.

Rollinger, J. M., T. Langer, et al. (2006). "Strategies for efficient lead structure discovery from natural products." Current Medicinal Chemistry **13**(13): 1491-1507.

Ross, J. A. and C. M. Kasum (2002). "Dietary flavonoids: Bioavailability, metabolic effects, and safety." Annual Review of Nutrition **22**: 19-34.

Roszkowski, W., J. Beuth, et al. (1989). "Blocking of Lectin-Like Adhesion Molecules on Pulmonary Cells Inhibits Lung Sarcoma L-1 Colonization in Balb/C-Mice." Experientia **45**(6): 584-588.

Roy, H., S. Bhardwaj, et al. (2006). "VEGF-A, VEGF-D, VEGF receptor-1, VEGF receptor-2, NF-kappa B, and RAGE in atherosclerotic lesions of diabetic Watanabe heritable hyperlipidemic rabbits." FASEB Journal **20**(12): 2159-2161.

Rusnati, M. and M. Presta (1996). "Interaction of angiogenic basic fibroblast growth factor with endothelial cell heparan sulfate proteoglycans - Biological implications in neovascularization." International Journal of Clinical and Laboratory Research **26**(1): 15-23.

Russ, M., R. Martinez, et al. (2006). "Naringenin is a novel inhibitor of Dictyostelium cell proliferation and cell migration." Biochemical and Biophysical Research Communications **345**(1): 516-522.

Sagar, S. M., D. Yance, et al. (2006). "Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer-Part 1." Current Oncology **13**(1): 14-26.

Samaranayake, H., A. M. Maatta, et al. (2010). "Future Prospects and Challenges of Antiangiogenic Cancer Gene Therapy." Human Gene Therapy **21**(4): 381-396.

Sane, D. C., L. Anton, et al. (2004). "Angiogenic growth factors and hypertension." Angiogenesis **7**(3): 193-201.

Sartippour, M. R., N. P. Seeram, et al. (2008). "Ellagitannin-rich pomegranate extract inhibits angiogenesis in prostate cancer in vitro and in vivo." International Journal of Oncology **32**(2): 475-480.

Sasamura, H., A. Takahashi, et al. (2004). "Antiproliferative and antiangiogenic activities of genistein in human renal cell carcinoma." Urology **64**(2): 389-393.

Scalbert, A. and G. Williamson (2000). "Dietary intake and bioavailability of polyphenols." Journal of Nutrition **130**(8S Suppl): 2073S-2085S.

Schafer, A. I. (1995). "Effects of Nonsteroidal Antiinflammatory Drugs on Platelet-Function and Systemic Hemostasis." Journal of Clinical Pharmacology **35**(3): 209-219.

Schindler, R. and R. Mentlein (2006). "Flavonoids and vitamin E reduce the release of the angiogenic peptide vascular endothelial growth factor from human tumour cells." Journal of Nutrition **136**(6): 1477-1482.

Schlessinger, J., A. N. Plotnikov, et al. (2000). "Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization." Molecular Cell **6**(3): 743-750.

Schluter, H. and W. Zidek (1993). "Application of Non-Size-Related Separation Effects to the Purification of Biologically-Active Substances with a Size-Exclusion Gel." Journal of Chromatography **639**(1): 17-22.

Seghezzi, G., S. Patel, et al. (1998). "Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: An autocrine mechanism contributing to angiogenesis." Journal of Cell Biology **141**(7): 1659-1673.

Serini, G.; L. Trusolino, et al. (1996). "Changes in integrin and E-cadherin expression in neoplastic versus normal thyroid tissue." Journal of the National Cancer Institute **88**(7): 442-449.

Serini, G., D. Valdembri, et al. (2006). "Integrins and angiogenesis: A sticky business." Experimental Cell Research **312**(5): 651-658.

Setchell, K. D. (2000). "Absorption and metabolism of soy isoflavones-from food to dietary supplements and adults to infants." Journal of Nutrition **130**(3): 654S-655S.

Setchell, K. D. R. (1998). "Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones." American Journal of Clinical Nutrition **68**(6): 1333s-1346s.

Setchell, K. D. R., N. M. Brown, et al. (2002). "Soy isoflavone glycosides do not cross the enterocyte intact: Quantitative importance of intestinal metabolism in their bioavailability." Journal of Nutrition **132**(3): 593s-595s.

Shao, Z. M., M. L. Alpaugh, et al. (1998). "Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by P21(WAF1/CIP1) induction, G(2)/M arrest, and apoptosis." Journal of Cellular Biochemistry **69**(1): 44-54.

Shao, Z. M., Z. Z. Shen, et al. (2002). "Curcumin exerts multiple suppressive effects on human breast carcinoma cells." International Journal of Cancer **98**(2): 234-240.

Shariat, S. F., R. F. Youssef, et al. (2010). "Association of Angiogenesis Related Markers With Bladder Cancer Outcomes and Other Molecular Markers." Journal of Urology **183**(5): 1744-1750.

Shibayama-Imazu, T., T. Aiuchi, et al. (2008). "Vitamin K(2)-mediated apoptosis in cancer cells: Role of mitochondrial transmembrane potential." Vitamin K **78**: 211-226.

Shin, E. Y., S. Y. Kim, et al. (2001). "c-Jun N-terminal kinase is involved in motility of endothelial cell." Experimental and Molecular Medicine **33**(4): 276-283.

Silva, M. M., M. R. Santos, et al. (2002). "Structure-antioxidant activity relationships of flavonoids: A re-examination." Free Radical Research **36**(11): 1219-1227.

Singh, R. P., G. Deep, et al. (2006). "Effect of silibinin on the growth and progression of primary lung tumours in mice." Journal of the National Cancer Institute **98**(12): 846-855.

Somekawa, Y., M. Chiguchi, et al. (2001). "Soy intake related to menopausal symptoms, serum lipids, and bone mineral density in postmenopausal Japanese women." Obstetrics and Gynecology **97**(1): 109-115.

Sosulski, F. W. and K. J. Dabrowski (1984). "Composition of Free and Hydrolyzable Phenolic-Acids in the Flours and Hulls of 10 Legume Species." Journal of Agricultural and Food Chemistry **32**(1): 131-133.

Staton, C. A., M. W. R. Reed, et al. (2009). "A critical analysis of current in vitro and in vivo angiogenesis assays." International Journal of Experimental Pathology **90**(3): 195-221.

Stewart, D. J., M. J. B. Kutryk, et al. (2009). "VEGF Gene Therapy Fails to Improve Perfusion of Ischemic Myocardium in Patients With Advanced Coronary Disease: Results of the NORTHERN Trial." Molecular Therapy **17**(6): 1109-1115.

Stoner, G. D., T. Chen, et al. (2006). "Protection against esophageal cancer in rodents with lyophilized berries: Potential mechanisms." Nutrition and Cancer-an International Journal **54**(1): 33-46.

Suja, K. P., A. Jayalekshmy, et al. (2004). "Free radical scavenging behavior of antioxidant compounds of sesame (Sesamum indicum L.) in DPPH center dot system." Journal of Agricultural and Food Chemistry **52**(4): 912-915.

Susanti (2011). Angiogenesis modulating activity of NOD factor-derived compounds and histidine-rich glycoprotein. John Curtin of Medical Research, Australian national University. **MPhil**.

Szymczak, M., M. Murray, et al. (2008). "Modulation of angiogenesis by omega-3 polyunsaturated fatty acids is mediated by cyclooxygenases." Blood **111**(7): 3514-3521.

Takasaki, M., T. Konoshima, et al. (2000). "Anti-tumour-promoting activity of lignans from the aerial part of Saussurea medusa." Cancer Letters **158**(1): 53-59.

Tammela, T., B. Enholm, et al. (2005). "The biology of vascular endothelial growth factors." Cardiovascular Research **65**(3): 550-563.

Taraboletti, G., S. D'Ascenzo, et al. (2002). "Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells." American Journal of Pathology **160**(2): 673-680.

Tenhagen, M., P. J. van Diest, et al. (2012). "Fibroblast growth factor receptors in breast cancer: expression, downstream effects, and possible drug targets." Endocrine-Related Cancer **19**(4): R115-R129.

Thaloor, D., A. K. Singh, et al. (1998). "Inhibition of angiogenic differentiation of human umbilical vein endothelial cells by curcumin." Cell Growth and Differentiation **9**(4): 305-312.

Tham, D. M., C. D. Gardner, et al. (1998). "Clinical review 97 - Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological, and mechanistic evidence." Journal of Clinical Endocrinology and Metabolism **83**(7): 2223-2235.

Tonnesen, M. G., X. D. Feng, et al. (2000). "Angiogenesis in wound healing." Journal of Investigative Dermatology Symposium Proceedings **5**(1): 40-46.

Tordera, M., M. L. Ferrandiz, et al. (1994). "Influence of anti-inflammatory flavonoids on degranulation and arachidonic acid release in rat neutrophils." Z Naturforsch C **49**(3-4): 235-240.

Touil, Y. S., A. Fellous, et al. (2009). "Flavonoid-Induced Morphological Modifications of Endothelial Cells Through Microtubule Stabilization." Nutrition and Cancer-an International Journal **61**(3): 310-321.

Tu, Y., C. Jeffries, et al. (2010). "Automated High-Throughput System to Fractionate Plant Natural Products for Drug Discovery." Journal of Natural Products **73**(4): 751-754.

Turner, N. and R. Grose (2010). "Fibroblast growth factor signalling: from development to cancer." Nature Reviews Cancer **10**(2): 116-129.

Umezawa, T. (2007). "Lignan and norlignan biosynthesis and biotechnology." Biotechnology and Sustainable Agriculture 2006 and Beyond: 341-343.

Van Miert, S., S. Van Dyck, et al. (2005). "Antileishmanial activity, cytotoxicity and QSAR analysis of synthetic dihydrobenzofuran lignans and related benzofuranns." Bioorganic and Medicinal Chemistry **13**(3): 661-669.

Vanholme, R., B. Demedts, et al. (2010). "Lignin Biosynthesis and Structure." Plant Physiology **153**(3): 895-905.

Ververidis, F., E. Trantas, et al. (2007). "Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes." Biotechnology Journal **2**(10): 1235-1249.

Walle, T. (2004). "Absorption and metabolism of flavonoids." Free Radical Biology and Medicine **36**(7): 829-837.

Walle, T., A. M. Browning, et al. (2005). "Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans." Journal of Nutrition **135**(1): 48-52.

Wallerath, T., G. Deckert, et al. (2002). "Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase." Circulation **106**(13): 1652-1658.

Walsh, K. R. (2006). Digestion and intestinal metabolism of soy isoflavonoids and isoflavonoid metabolites, The Ohio State University. **PhD**.

Wang, H. J. and P. A. Murphy (1994). "Isoflavone Composition of American and Japanese Soybeans in Iowa - Effects of Variety, Crop Year, and Location." Journal of Agricultural and Food Chemistry **42**(8): 1674-1677.

Wang, H. J. and P. A. Murphy (1994). "Isoflavone Content in Commercial Soybean Foods." Journal of Agricultural and Food Chemistry **42**(8): 1666-1673.

Wang, L. S., A. A. Dombkowski, et al. (2011). "Mechanistic Basis for the Chemopreventive Effects of Black Raspberries at a Late Stage of Rat Esophageal Carcinogenesis." Molecular Carcinogenesis **50**(4): 291-300.

Wang, T. T. Y., N. Sathyamoorthy, et al. (1996). "Molecular effects of genistein on estrogen receptor mediated pathways." Carcinogenesis **17**(2): 271-275.

Wani, M. C., H. L. Taylor, et al. (1971). "Plant Antitumour Agents .6. Isolation and Structure of Taxol, a Novel Antileukemic and Antitumour Agent from Taxus-Brevifolia." Journal of the American Chemical Society **93**(9): 2325-2327.

Wase, N. V. and P. C. Wright (2008). "Systems biology of cyanobacterial secondary metabolite production and its role in drug discovery." Expert Opinion on Drug Discovery **3**(8): 903-929.

Wasson, A. P., K. Ramsay, et al. (2009). "Differing requirements for flavonoids during the formation of lateral roots, nodules and root knot nematode galls in Medicago truncatula." New Phytologist **183**(1): 167-179.

Weis, S. M. and D. A. Cheresh (2011). "Tumour angiogenesis: molecular pathways and therapeutic targets." Nature Medicine **17**(11): 1359-1370.

Whetten, R. and R. Sederoff (1995). "Lignin Biosynthesis." Plant Cell **7**(7): 1001-1013.

Williamson, G. and C. Manach (2005). "Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies." American Journal of Clinical Nutrition **81**(1 Suppl): 243S-255S.

Winkel-Shirley, B. (2002). "Biosynthesis of flavonoids and effects of stress." Current Opinion in Plant Biology **5**(3): 218-223.

Witters, L. A. (2001). "The blooming of the French lilac." Journal of Clinical Investigation **108**(8): 1105-1107.

Witters, L. M., S. M. Santala, et al. (2003). "Decreased response to paclitaxel versus docetaxel in HER-2/neu transfected human breast cancer cells." American Journal of Clinical Oncology-Cancer Clinical Trials **26**(1): 50-54.

Wu, S. H., L. Yang, et al. (2008). "Multi-channel counter-current chromatography for high-throughput fractionation of natural products for drug discovery." Journal of Chromatography A **1180**(1-2): 99-107.

Wu, W. B., H. S. Chiang, et al. (2007). "Inhibitory effect of lycopene on PDGF-BB-induced signalling and migration in human dermal fibroblasts: a possible target for cancer." Biochemical Society Transactions **35**: 1377-1378.

Xu, X., H. J. Wang, et al. (1994). "Daidzein Is a More Bioavailable Soymilk Isoflavone Than Is Genistein in Adult Women." Journal of Nutrition **124**(6): 825-832.

Yamamoto, T., M. Yoshimura, et al. (2004). "Anti-allergic activity of naringenin chalcone from a tomato skin extract." Bioscience Biotechnology and Biochemistry **68**(8): 1706-1711.

Yang, S. H., J. K. Lin, et al. (2003). "Anti-angiogenic effect of silymarin on colon cancer LoVo cell line." Journal of Surgical Research **113**(1): 133-138.

Yu, X. P., J. D. Zhu, et al. (2012). "Anti-angiogenic genistein inhibits VEGF-induced endothelial cell activation by decreasing PTK activity and MAPK activation." Medical Oncology **29**(1): 349-357.

Zava, D. T. and G. Duwe (1997). "Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro." Nutrition and Cancer International Journal **27**(1): 31-40.

Zhang, F. Y., G. J. Du, et al. (2009). "Naringenin Enhances the Anti-Tumour Effect of Doxorubicin Through Selectively Inhibiting the Activity of Multidrug Resistance-Associated Proteins but not P-glycoprotein." Pharmaceutical Research **26**(4): 914-925.

Zhang, W., X. Wang, et al. (2008). "Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholesterolaemic subjects." British Journal of Nutrition **99**(6): 1301-1309.

Zhao, T., W. Zhao, et al. (2011). "Acidic and basic fibroblast growth factors involved in cardiac angiogenesis following infarction." International Journal of Cardiology **152**(3): 307-313.

Zhou, Y. J., Y. E. Liu, et al. (2009). "Vitexins, Nature-Derived Lignan Compounds, Induce Apoptosis and Suppress Tumour Growth." Clinical Cancer Research **15**(16): 5161-5169.

Zi, X., D. K. Feyes, et al. (1998). "Anticarcinogenic effect of a flavonoid antioxidant, silymarin, in human breast cancer cells MDA-MB 468: induction of G1 arrest through an increase in Cip1/p21 concomitant with a decrease in kinase activity of cyclin-dependent kinases and associated cyclins." Clinical Cancer Research **4**(4): 1055-1064.

"Soul of all souls, life of all life - you are That.
Seen and unseen, moving and unmoving - you are That.
The road that leads to the City is endless;
Go without head and feet
and you'll already be there
What else could you be? - you are That."

Rumi